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13. ABSTRACT (Maximum 200) The goal of this project has been to improve the detection and treatment of breast cancer by characterizing vasopressin (VP) gene expression by this disease and determining the nature and role of products generated through this expression. The VP gene is expressed by seemingly all breast cancers and by all DCIS, and this information coupled with an absence of VP gene-related products from fibrocystic disease potentially provides us with a new diagnostic test for distinguishing both breast cancer and DCIS from atypical hyperplasia. Studies on cell trafficking have shown that almost all protein processing is outside of secretory vesicles, the major processing enzymes (CPE, PC1/3, PC2, and PAM) are expressed, and components of GRSA comprise both 20 KDa and 40 KDa VP-related proteins. Meioties on both of these proteins are potential targets for immunotherapy. Partial structures for these proteins have been determined, and Abs against them have been generated. Substantial evidence supports the mitogenic actions of VP on breast cancer, and we have obtained the structure for a new putative hVACM VP receptor expressed by breast cancer cells. Breast cancer expression of all other VP receptor subtypes (V1a, V1b, V2, and an abnormal V2) has been confirmed. Recently we have achieved the complete sequencing of the open reading frames for V1a, V1b, and V2 receptors of breast cancer, and discovered that all normal structures are present. With remaining funds we intend to ascertain the effectiveness of our Abs to target breast cancer cells in vivo.			
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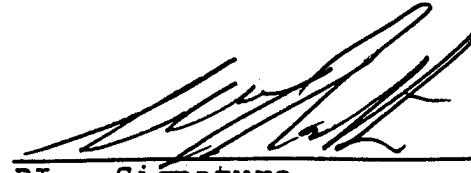

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(5) Introduction

The overall objective of this project was to improve the detection and treatment of breast cancer by evaluating vasopressin gene-related products as tumor marker substances in hyperplastic breast disease, by characterizing the nature and regulation of the vasopressin gene and its products in breast cancer, and by determining the potential usefulness of vasopressin gene-related products on tumor membranes as targets for immunotherapy. It sought to test the hypothesis that all breast tumors produce vasopressin as an autocrine growth factor, *in situ*, and that this property can be effectively utilized not only to elucidate the pathobiology of this cancer, but also to identify precancerous tissue and develop more successful treatments.

In hypothalamic neurons, vasopressin gene expression leads to the formation of a 750 bp mRNA and the subsequent generation of a 20 KDa precursor that undergoes intragranular enzymatic processing to form vasopressin (VP), vasopressin-associated human neurophysin (VP-HNP), and vasopressin-associated glycopeptide (VAG). All three of these products are released into the circulation by exocytosis. None of these products become components of the plasma membrane of neurons.

We have shown that the vasopressin gene of chromosome 20 is expressed by apparently all breast tumors, but not by normal breast tissue (*North et al., 1995). This indicates that in the mammary gland, the expression of the vasopressin gene is a feature unique to tumor cells, a feature common to all hyperplastic tissues, or a feature shared only by tumor cells and their progenitors. The first and third of these possibilities raised the potential use of this expression as a marker of carcinogenesis, and/or forecaster of imminent disease. We therefore conducted a survey of the incidence of vasopressin gene expression in fibrocystic disease, and this work has been accepted, with modification, for publication in *Endocrine Pathology*. No evidence for gene expression could be found for all cases of fibrocystic disease examined, including atypical intraductal hyperplasia. In our study, three individuals with benign breast disease went on to develop breast cancer. Taken together, these findings indicate vasopressin gene expression is not a marker of cellular proliferation in the breast, nor a marker of cancer progenitor cells in benign breast disease (*Fay et al., 1997). This leads us to the conclusion that vasopressin gene expression in the breast is likely to be solely associated with the process of carcinogenesis. Therefore, it would seem the vasopressin gene is an oncogenic marker of breast cancer. We have recently confirmed this through studying vasopressin gene expression in cases of carcinoma *in situ* (DCIS). All of 12 cases of DCIS studied showed a clear expression of the vasopressin gene, and we are currently investigating the possibility of using this finding to develop a diagnostic test for distinguishing atypical hyperplasia from DCIS (see Body of this report).

Expression of the vasopressin gene in breast cancer leads to the formation of unique gene-related products, some of which become associated with the plasma membrane of tumor cells. Because these membrane-associated products react with antibodies raised against human vasopressin-associated glycopeptide (VAG), we have referred to them as **GRSA** (Glycopeptide Related cell Surface Antigen). Because they are located at the cell membrane of the tumor cells, we have demonstrated they can be targeted, *in vitro*, with antibodies to VAG. This raises the possibility they can be utilized for targeting tumors in patients through immunotherapy. We have excellent indirect evidence that strengthens this possibility. Breast cancer uniquely shares the feature of membrane expression of vasopressin gene-related products with small-cell carcinoma of the lung (SCCL), and we have shown we can successfully target these products in SCCL patients using radioiodinated and Indium-labeled antibodies (*North et. al, 1989, *North and Yu, 1993).

What is the nature of GRSA? The VP mRNA and protein products that arise in breast cancer through expression of the vasopressin gene appear to be both structurally normal and abnormal (see Body of this report). We had anticipated this possibility because we (and others) have earlier shown that abnormal and normal forms co-exist in SCCL (*North et. al, 1983; Rosenbaum et. al, 1990; *North and Yu, 1993). There appear to be two VPmRNAs in both breast cancer and SCCL. One of these is sequentially almost identical to that in human hypothalamic neurons, while the other is extended by 600 base pairs at the 5' end of the reading frame. The VPmRNAs of both types of tumors give rise to proteins of 40 KDa and 20 KDa as prominent forms, although the proteins of breast cancer appear to show some structural

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differences to those of SCCL (*North et al., 1995). The 20 KDa form of SCCL is almost identical to the provasopressin of hypothalamic neurons. Both 40 KDa and 20 KDa proteins of SCCL become incorporated into the cell membrane as cell-surface antigens. Studies to fully characterize the two VPmRNAs of breast cancer are still in being performed. We have recently shown that both 40 KDa and 20 KDa proteins of this tumor type represent GRSA at tumor cell surfaces (see Body of this report).

In normal hypothalamic neurons, 20 KDa provasopressin is processed by proteolysis that is thought to involve at least four enzymes. That such proteolysis also occurs in breast cancer is evidenced by our preliminary findings that most patients with breast cancer have inappropriately high plasma levels of vasopressin, and elevated levels of VAG (unpublished data). Breast cancer can therefore be classified as neuroendocrine in nature. Because of this, we performed studies that demonstrated the presence of the key processing enzymes, carboxypeptidase E, and prohormone convertases PC2 or PC1/3, and PAM, in the two breast cancer cell lines MCF7 and ZR-75-1.

Why is vasopressin produced by breast cancer? One answer to this question is that vasopressin serves as an autocrine growth factor for these tumors. Vasopressin is already known to act as a growth factor/growth modulating agent in SCCL lines where it promotes calcium mobilization and clonal growth (Hong and Moody, 1991; Sethi and Rozengurt, 1991, Cassoni et al., 1994, 1996, 1997). Over the last three years we reported that vasopressin can promote calcium mobilization in two breast cancer cell lines, ZR-75-1 and T47D, and can dramatically influence the cytoskeleton of ZR-75-1. These finding are supported by previous studies on a dimethylbenzathrene-induced rat mammary tumor (Monaco et al., 1978; Monaco et al., 1980; Guilon et al., 1986; Kirk et al., 1986; Woods and Monaco, 1988), human MCF7 breast cancer cells (Taylor et al., 1990), and on another breast cancer cell line (Bunn et al., 1992). Choi et al. (1994) were also able to show that vasopressin promotes growth of mammary tumors in transgenic mice. These actions of vasopressin have prompted us to investigate the nature of vasopressin receptors on breast cancer cells. Four vasopressin receptors have been indentified in other cells and have been cloned (Birnbaumer et al., 1992; Hirasawa et al., 1994; Sugimoto et al., 1994; Thibonnier et al., 1994; Burnatowska-Hledin et al., 1995, *Fay et al., 1994, 1996; *North et al., 1997a, 1997b). These are known as vasopressin V_{1a}, V_{1b}, and V₂, receptors plus vasopressin-activated calcium-mobilizing receptor (VACM1). Although an investigation of vasopressin receptors and the growth promotional activities of vasopressin may seem to fall outside of intentions enunciated in the original proposal, we believe they nevertheless address the body of the hypothesis originally advanced in the proposal and fall within the goals of Technical objectives 2 and 3. It is believed that such an investigation could not only explain the seemingly universal expression of the vasopressin gene in breast tumors, but also lead to an additional number of effective therapies. Therefore, over the course of this year we have now completed the sequencing of the entire open reading frames (ORFs) of vasopressin V_{1a}, V_{1b}, and V₂ receptor mRNAs of the breast cancer cell line MCF-7 and have prepared a manuscript for submission to the journal, Molecular Endocrinology (see Appendix).

(6) Body

Technical Objective 1: Vasopressin gene-expression in breast hyperplasia as a predictor of cancer (Task 1 in Statement of Work). Breast Cancer/Carcinoma in situ/hyperplasia.

This objective has been satisfied. We also report on our recent discovery that the vasopressin gene is expressed by all carcinoma in situ examined and the implications of this finding. These recent findings have not yet been published. Also, for the sake of clarity, we include below a summary of all earlier reported findings. Our findings taken together show that vasopressin gene expression is a marker of oncogenic transformation in breast tissues.

Breast Cancer: We performed immunohistochemistry on 19 breast cancers representing a variety of tumor subtypes using antibodies directed against different moieties of the vasopressin precursor structure as indicated in Figure 1, below. These comprised rabbit polyclonal antibodies that recognize arginine vasopressin (anti-VP), the tripeptide bridge region of the precursor (anti-ProVP), and the carboxyl region of vasopressin-associated human glycopeptide (anti-VAG); and mouse monoclonal antibodies that

recognize an amino terminal portion of vasopressin-associated human neurophysin (anti-VP-HNP). Western Blot analysis was performed on protein extracts from an additional 12 breast tumors.

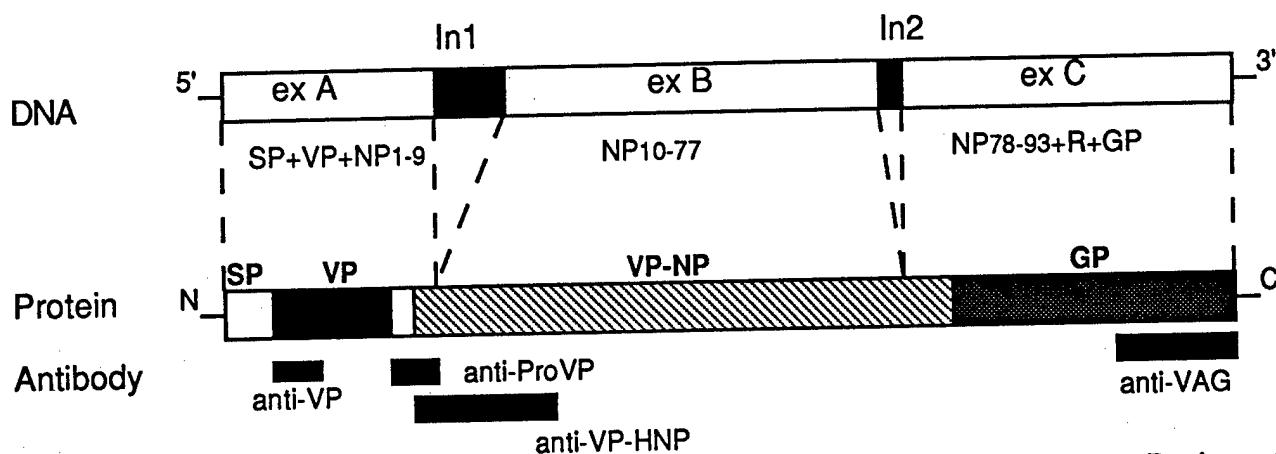


Figure 1. Illustration depicting the structures of the vasopressin gene and protein precursor. Regions of the precursor are blocked out against which Abs, used in immunohistochemistry of breast cancer, are directed.

As shown in Table 1, while VP-related proteins were not detected in normal breast tissues, immunohistochemistry revealed the presence of VP and VAG in all neoplastic cells of all tumor tissues examined. ProVP was evident in 11 of 14 tumors while VP-HNP was evident in only one of 19 tumors examined.

Table 1. Presence of vasopressin gene related products in human breast cancer

cancer subtype	VP gene related antigens*			
	VP	ProVP	VP-HNP	VAG
Infiltrating ductal	na	na	-	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	+	+	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	-	-	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	na	-	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	-	-	+
Infiltrating ductal	+	-	-	+
Colloid	+	+	-	+
Colloid	+	na	-	+
Colloid	+	+	-	+
Colloid	+	+	-	+
Infiltrating tubular	+	na	-	+
Infiltrating tubular	+	+	-	+
Infiltrating lobular	+	na	-	+
Total positive	18/18	11/14	1/19	19/19

*Positive (+) or negative (-) immunoreactivity using antibody preparations and the ABC procedure.
na = not attempted.

However, Western blot analysis for all 12 fresh-frozen tumor samples showed the presence of two proteins of 42 KDa and 20 KDa, that were both immunoreactive with, not only antibodies against VP and VAG, but also those against VP-HNP (anti-ProVP were not used). The vasopressin precursor of hypothalamic tissues is 20 KDa in size. These findings provided evidence that the vasopressin gene is expressed as a selective feature of all breast cancers. This expression apparently gives rise to an abnormally large vasopressin-related protein, and one protein of normal size with possible modifications in the neurophysin region making it less immunoreactive with anti-VP-HNP. Both proteins represent potential markers for tumor detection and potential targets for immunotherapy.

Fibrocystic Disease: In order to examine if vasopressin gene expression was a possible predictor of disease, we performed a survey of the incidence of vasopressin gene expression in fibrocystic disease, and this work has now been accepted, pending revision, for publication in *Endocrine Pathology*. In this study, we used immunohistochemistry and antibodies against vasopressin (anti-VP) and vasopressin-associated glycopeptide (anti-VAG) to examine formalin-fixed biopsy specimens taken from 17 patients, with various forms of benign breast disease, who were seen at Dartmouth Hitchcock Medical Center between 1975 and 1984. These specimens were selected without any knowledge of follow-up, and included 4 cases of atypical ductal hyperplasia, 6 cases of fibrocystic disease with intraductal hyperplasia, 2 cases of fibrocystic disease with papilloma, 1 case of fibrocystic disease with bilateral mammary hyperplasia, and 4 cases of typical fibrocystic disease. Diagnosis from pathology reports was confirmed by examining hematoxylin- and eosin-stained sections. The results of these studies are illustrated in Table 2, and demonstrate that in all cases of benign breast disease examined there was negative staining for both vasopressin and vasopressin-associated glycopeptide. They indicate that the vasopressin gene is *not* expressed in benign breast disease, and this is in dramatic contrast to what was found for human breast carcinoma using these same antibodies (Table 1). At the completion of the study, it was discovered that three of the individuals with benign breast disease went on to develop breast carcinoma. Although preliminary, these data taken together indicate that (i) expression of vasopressin gene related products is not a marker of cellular proliferation in the breast, (ii) expression of vasopressin gene-related products is associated with the process of carcinogenesis, and (iii) expression of vasopressin gene-related products is not a marker of precancerous cells in benign breast disease.

Table 2. Absence of vasopressin gene-related products from benign breast fibrocystic conditions

Subtype	VP gene-related antigens*	
	VP	VAG
Fibrocystic Disease	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Atypical Intraductal Hyperplasia	-	-
Atypical Intraductal Hyperplasia	-	-
Atypical Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Papilloma	-	-
Fibrocystic Disease with Intraductal Papilloma	-	-
Fibrocystic Disease with Bilateral Mammary Hyperplasia	-	-
Total Positive	0/16	0/16

*Positive (+) or negative (-) immunoreactivity using ABC immunohistochemistry

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Carcinoma in situ: We used immunohistochemistry with anti-VAG antibodies to examine vasopressin gene expression in pre-invasive carcinoma. Blocked out biopsy samples of twelve cases of carcinoma in situ, six of which have been clearly identified as being of the comedo variety with abnormal cells and extensive necrotic areas, were investigated. All twelve cases (Table 3) showed positive staining with anti-VAG demonstrating for this small sampling that vasopressin gene expression is commonly associated with breast carcinoma in situ (Figure 2). Of the DCIS samples, the comedo variety gave the most intense staining.

Table 3. Presence of vasopressin gene-related products in carcinoma in situ

Subtype	<u>VP gene-related antigen*</u> VAG
Carcinoma in situ, non-comedo	+
"	+
"	+
"	+
"	+
"	+
Carcinoma in situ, comedo	+
"	+
"	+
"	+
"	+
"	+
Total Positive	12/12

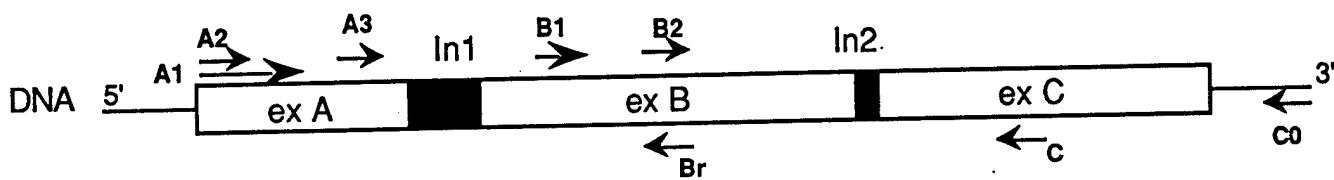
*Positive (+) or negative (-) immunoreactivity using ABC immunohistochemistry

Figure 2. Carcinoma in situ stained using the ABC immunohistochemical method with Abs against VAG

The above results indicate that ABC immunohistochemistry with our antibodies to VAG can clearly distinguish atypical ductal hyperplasia from carcinoma in situ, a distinction currently difficult to make using other available methods. This distinction is important because a diagnosis of atypical hyperplasia has no follow-up, while carcinoma in situ is generally followed-up with ablative surgery. We are therefore intending to further test this finding by embarking on a screening study that will compare evaluation by histochemical analysis alone with an evaluation that uses both histochemistry and VAG immunohistochemistry.

Technical Objective 2: Characterization of vasopressin gene expression by breast cancer cells (Tasks 2 and 3 in Statement of Work).

The data discussed in this section are largely unpublished.



Structure of Human vasopressin gene and locations of some designed PCR primers
Figure 3

We have established for breast cancer cells that there is abnormal, in addition to normal, production of vasopressin. Abnormal protein forms constituting GRSA might to be generated from one normal and one abnormal gene. RT-PCR, cloning, and sequencing studies on messages from the vasopressin gene of MCF7, T47D, and ZR-75-1 cells have now shown that there appear to be at least two VPmRNAs expressed in breast cancer, one from a 'normal' gene and the product of normal splicing, the second either from a 'normal' gene and the product of alternate splicing or from an abnormal gene having insertions in exon A. The ten primers used in studies are illustrated in the figure above and described in the following table:

Table 4. Forward and reverse primers designed for RT-PCR amplification of human vasopressin gene fragments from human breast cancer cells

Forward primer	Length	Nucleotides	Exon	Sequence
A1	21	269-289	1	5'-cttctccgcgtgctactt-3'
A2	18	269-286	1	5'-cttctccgcgtgcta-3'
A3	21	321-341	1	5'-atgtccgacctggagctgaga-3'
IN	21	1504-1524	intron 1	5'-gtcatccaagaaaccaagggtg-3'
B1	25	1751-1775	2	5'-tgcttcggcccaagcatctgctg-3'
B2	22	1830-1851	2	5'-tgccaggaggagaactacgtc-3'
Reverse primer				
INR	20	1517-1536	intron 1	5'-agatctgctggcaccttgg-3'
Br	22	1830-1851	2	5'-gcaggtagttctccctggga-3'
C	22	2152-2173	3	5'-agcaacgcccacgcagctggacg-3'
C0	25	2231-2255	3	5'-taggcgtcggctggcggctcga-3'

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Normal-sized VPmRNA fragments of 313 bp using A3C were obtained from three cell lines. These have been partially sequenced and shown to have a sequence very similar to the VPmRNA found in hypothalamic neurons. We also isolated, and successfully reamplified (but have not yet sequenced) an RT-PCR product(s), from all three cell lines using the specific primers A1 and C, that is 600 bases larger than that predicted from the structure of VPmRNA. Such a structure could represent a VPmRNA that have retained a 600 base portion of intron 1 through alternative splicing (the entire intron 1 segment contains 1373 bases). If the 5' sequence of the product confirms it translates a protein with the N-terminus of provasopressin, it will offer one explanation for the 40,000 dalton species of breast cancer because an extra 600 bases represents an additional 200 amino acid residues. Adding 200 amino acid residues to the 20,000 dalton provasopressin would give a protein of 40,000 daltons. Since antibodies recognize the exon B (at least in Western analyses) and exon C regions of the protein (North et al., 1995) the intronic insertion would not apparently cause a reading frame shift. The structure of the enlarged form will now be checked through reamplification using both A2 and C primers, and A3 and C primers. If the additional 600 bases in A1C are from intron 1, we expect in all cases, reamplified products that are approximately 600 bases larger than predicted from normal VPmRNA. However, if products of normal size are produced this will suggest the enlarged form represents an abnormal vasopressin gene having a 600 base insertion in the exon A region. This insertion would be between bases corresponding to the vasopressin and neurophysin structures. Structures A2 and A3 are only separated by 35 bases in normal VPmRNA. While a definitive answer regarding the enlarged form will be best provided through cloning and DNA sequencing, the planned exercise will enable us to eliminate the possibility of alternative co-existing forms. Use of primers B1, B2, C and Co will likewise enable us to discover if forms extended in the exon B and/or exon C region exist in breast cancer cells (as found by us in SCCL), while use of the forward IN and reverse INR primers will allow us, when used with B and A primers, to obtain shortened RT-PCR products for sequencing if regions of intron 1 are indeed included in the abnormal VPmRNA structure. All of these primers have already been used somewhat successfully by us in sequencing VPmRNA forms from SCCL (unpublished). However, two abnormal VPmRNA structures found by us for SCCL have recently been entered into the GENEbank with accession numbers

We have also made efforts to perform Edman sequencing on purified samples of GRSA proteins. We have decided to concentrate our studies on protein obtained from the cell line MCF-7 and used these cultured cells as the protein sources. Purification employed pH-salt separations, molecular sieve chromatography, and affinity chromatography on columns of Antivasopressin-Sepharose. Our antivasopressin monoclonal antibody, DEN1, was used to generate such an affinity resin. Protein mixes from affinity chromatography were S-alkylated and then separated using SDS-PAGE. We then intended performing solid-phase sequencing. However, both 20 KDa and 40 KDa proteins appeared to have blocked N-terminal residues, so, as yet, no sequence information could be found for GRSA proteins using this approach.

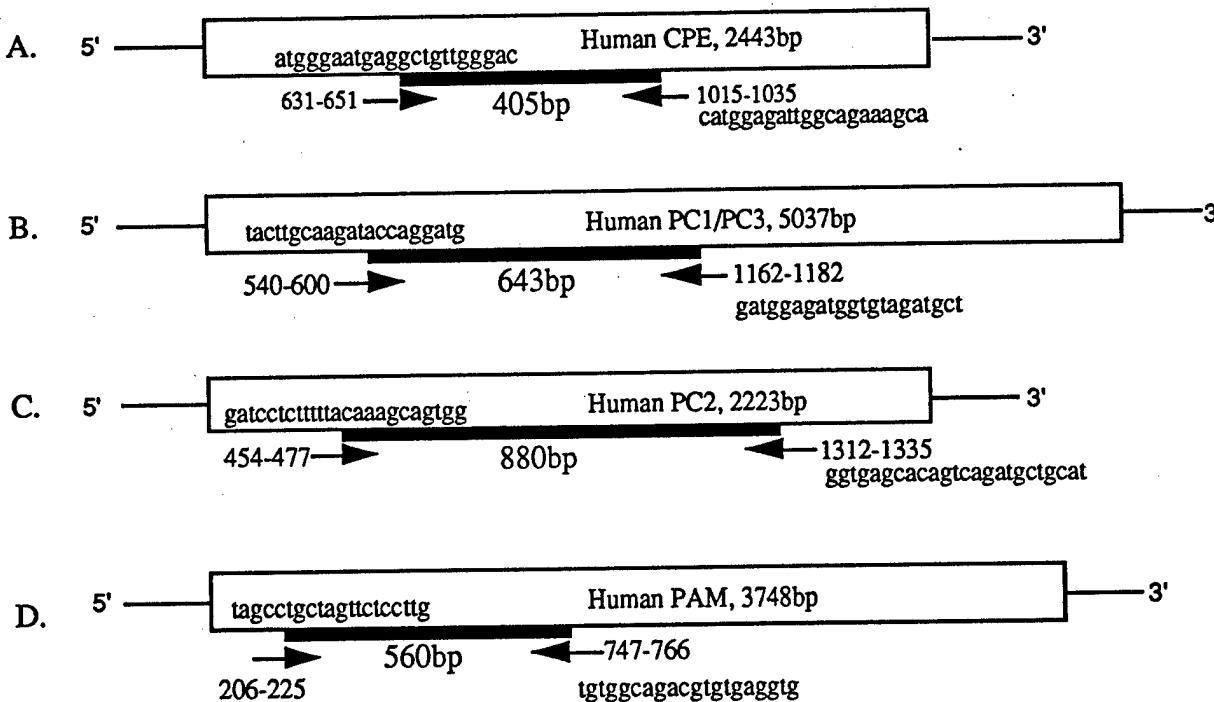
PCR studies on DNA preparations from breast cancer cell lines have also been conducted using a mixture of specific primers for the vasopressin gene and oxytocin genes. This is because a published study (Morris et al., 1995) has indicated that some hypothalamic neurons in rats can express protein products that are a composite of provasopressin and pro-oxytocin through a cross-over between the vasopressin and oxytocin genes on chromosome 20. We have established that there is no evident cross-over between the vasopressin and oxytocin genes in breast cancer.

Studies were also performed that examined sub-cellular trafficking in ZR-75-1 breast cancer cells (unpublished data).

Sucrose-gradient sub-fractionation of these cells (10^8 cells/batch) was carried out and an evaluation conducted by Western analysis and by RIA (VP, VP-HNP, VAG). This evaluation revealed that approximately 80% of both the 20 KDa and 40 KDa proteins are located in the plasma membrane. Of the remaining 20%, most (90%) is found outside secretory granules, and approximately 10% is within these granules. The procedures employed were found by us to preserve granules from hypothalamic neurons with >90% of vasopressin gene-related products located in the granular fraction. Hence, either the granules of breast cancer are more susceptible to rupture, or only a small percentage (< 2%) of translated protein is potentially processed to active hormone within these granules and then secreted. This implies that packaging is limited and most protein in breast cancer cells is destined for agranular targeting to the

plasma membrane. Both 20 KDa and 40 KDa proteins were found in the granular fraction of cells. This indicates that the 40 KDa product shows a capacity similar to the 20 KDa product to be packaged in the Golgi apparatus. This study indicates that the limited processing of 20 KDa and 40 KDa vasopressin gene-related proteins in breast cancer is largely due to limited packaging of translated material, rather than to an absence of processing enzymes. An almost identical trafficking pattern was found for SCCL cells in culture and reported on last year.

The breast cancer cell lines MCF7 and ZR-75-1 were examined for the expression of mRNAs for the processing enzymes carboxypeptidase E (CPE), prohormone convertases PC2 and PC1 (or PC3), and PAM, using RT-PCR, cloning, and sequencing. These studies are not yet published. The primer pairs used in these studies are depicted in figure below.



Primers designed for amplification cDNA fragments of carboxypeptidase E(CPE)(A), prohormone convertases(PC)1/3(B), PC2(C) and peptidylglycine alpha-amidating monooxygenase(PAM)(D) from breast cancer cell line MCF-7.

RT-PCR studies on CPE provided amplified products of the size predicted from previously published studies on anterior pituitary cells using polyA⁺RNA from both cell lines. These products for MCF-7 were reamplified, cloned and sequenced to provide structures identical to those published for this enzyme. In RT-PCR studies on PC2 we have so far only been able to amplify a product using polyA⁺RNA from MCF7. We then investigated if mRNA for PC1/3 was expressed in MCF7 and ZR-75-1. However, RT-PCR failed to show that this mRNA was expressed in either cell line. We subsequently performed RT-PCR for the enzyme employing the more sensitive 'platinum' Taq polymerase and were then able to demonstrate the enzyme PC1/3 is expressed by MCF-7 cells. The expression of all these enzymes by breast cancer have now been confirmed using available antibodies against CPE, PC1, PC2, and PAM in Western analysis. These antibodies were provided to us through the generosity of Dr. Lloyd Fricker of Albert Einstein Medical School, and of Drs. Betty Eiper and Richard Mains of Johns Hopkins.

Our results therefore show that at least four of the enzymes necessary for processing provasopressin to active hormone, neurophysin, and glycopeptide, are present in some breast cancer cell lines. This finding confirms conclusions expressed above that a failure of breast cancer cells to process vasopressin precursor proteins in the same manner or extent as central neurons is probably due to differences in sub-cellular packaging rather than to an absence of any of the enzymes necessary for processing.

Technical Objectives 3: Identification of factors regulating the production of GRSA by breast cancer; and 4: Determination of the binding properties for antibodies of GRSA and other vasopressin gene-products at tumor cell surfaces (Tasks 4 and 5 of Statement of Work).

We were unable to commence studies designed to satisfy these technical objectives, but expect to complete some of these studies with carry-over funding during the next twelve months. We performed studies on the regulation of vasopressin gene-expression in SCCL as part of another ongoing project, so all of the methods are at hand to enable us to proceed without pause.

Cloning of a novel calcium-mobilizing receptor from cancer cells (NCI H146 SCCL cells and MCF7 breast cancer cells)

Two years ago we described our ability to demonstrate, for breast cancer cells, the expression of mRNA for the novel vasopressin receptor, called VACM, using RT-PCR and primers designed from the structure of the rabbit form of this protein. Since that time the structure of a human clone of VACM from placenta was published by a British research team (Byrd et al., 1997, Stankovic et al., 1997). In order to study the role of this putative receptor in breast cancer, we generated a cDNA clone from human cancer cells. Initial efforts were focused on the small-cell carcinoma cell line NCI-H146, because we had obtained signal transduction data the presence of functional VACM protein in these cells. However, we then obtained 5' and 3' RACE products covering the entire open reading frame of mRNA for the protein from MCF7 breast cancer cells. The primers employed in RACE are given in Table 6, below:

TABLE 6: Primers used for 5' and 3' RACE of VACM from MCF7 breast cancer cells

3'-PCR RACE primers

1432(forward)	5' gaa-tgg-cta-aga-gaa-gtt-ggt-atg 3'
138 (reverse)	5' ttg-ttt-ttg-taa-ggt-aag-gca-gag 3'

5'-PCR RACE primers

5' ATG (forward)	5' tcc-aag-tta-aag-aac-atg-gcg 3'
2082 (reverse)	5' tct-tct-ctc-atc-ctt-tct-gta-gtg 3'

The isolated VACM clone for NCI-H146 contains an open reading frame of 2,343 nucleotides and encodes a protein of a predicted size of 781 amino acids. Analysis programs failed to identify hydrophobicity regions of sufficient confluence to classify them as transmembrane regions. The following motifs were identified to be present in the protein structure: two protein kinase A phosphorylation domains (Thr 427 and Ser 731); 15 protein kinase C phosphorylation domains; a Tyrosine phosphorylation domain (Tyr 207); two myristylation sites between residues 180 and 185, and 664 and 669; and three glycosylation sites at Asn 145, Asn 289, and Asn 566. Although these findings are unpublished we have recently submitted a manuscript on the structure of HVACM in H146 cells to Cancer Research. Our cDNA sequence for what we are now referring to as HVACM from human cancer cells has been submitted to the GENEBank by us and has been assigned the accession number AF017061. A complete copy of the GENEBank submission is included in the appendix of this progress report. The availability of cloned HVACM should now allow us to examine in detail the expression of this putative vasopressin receptor, and determine its role in the vasopressin-induced mitogenesis of breast

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cancer. We have also had antibodies made to HVACM, and these will be used to examine the incidence and distribution of the protein in breast cancer from our archival library.

Vasopressin-induced phosphorylation (activation) of mitogen-activated protein kinase (MAPK).

We earlier reported that vasopressin can activate MAPK in breast cancer cells, and we have recently tried to provide quantitative data on MCF7 breast cancer cells using a fluorescence Western Blot procedure from ECL with a Molecular Dynamics Fluorimager. Two antibodies preparations employed recognize dually phosphorylated MAPK p42/p44 (activated MAPK), and MAPK regardless of phosphorylation status. Treatments with vasopressin and a vasopressin V1 antagonist for 5 and 15 minutes were compared with controls using Imagequant software. Data obtained support an increase in MAPK activation at 5 minutes,

but not at 15 minutes, and this increase could only be demonstrated for the p44 MAPK isoform. These data have not yet been published.

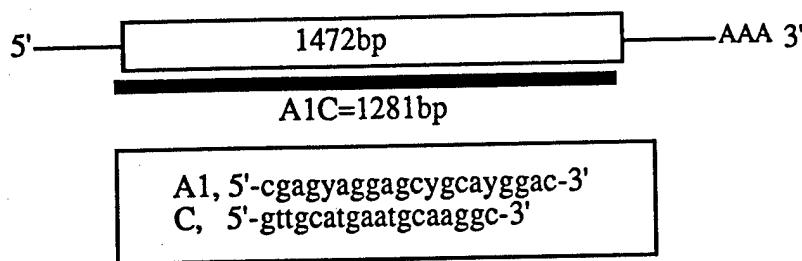
Sequencing of the vasopressin V1 and V2 receptor subtypes of breast cancer cells.

We earlier reported on the presence of vasopressin V_{1a} , V_{1b} , and V_2 receptors, and oxytocin receptors in breast cancer cell lines BT 549, MCF7, MDA, MB-231, T47D, and ZR-75 using specific primers and RT-PCR. We have now obtained sequences for the entire open reading frames (ORFs) of mRNAs for the all vasopressin receptors (three normal and one abnormal) for the MCF-7 breast cancer cell line. Complete sequence information on the open reading frames of all of these receptors produced by small-cell carcinoma of the lung were earlier published and submitted to the Genebank with accession numbers AF030625, AF030512, AF030626, and AF032388. We have now approached the gene bank about submitting the breast cancer receptor data.

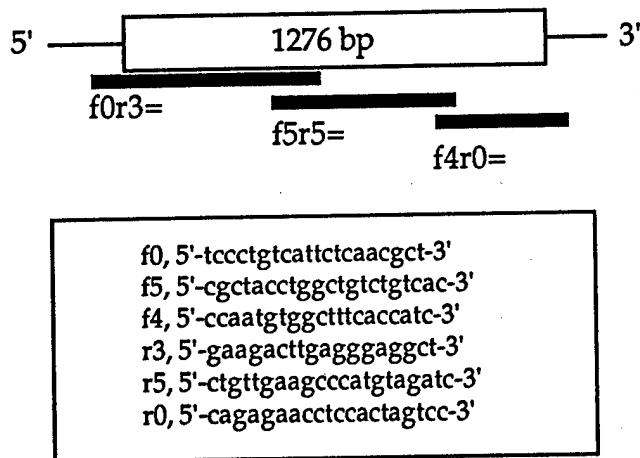
Using forward and reverse primers selected to provide overlapping sequences covering the entire open reading frames (ORFs) of vasopressin V_{1a} , and V_{1b} mRNAs, RT-PCR of poly(A)⁺ RNA preparations from MCF-7 cells yielded, in each case, a single product of the expected size predicted from the cDNA for the human forms of these receptors derived from liver (Thibonnier et al., 1994) or blood vessels (Hirasawa et al., 1994), from pituitary (Sugimoto et al., 1994; de Keyzer et al., 1994); and from small-cell lung cancer (North et al., 1998). All of the products were generated from RNA and not DNA template because no intronic segments that these products spanned were evident. The product obtained for the V_{1a} receptor (1472 bp), and the three obtained for the V_{1b} receptor are shown in the figure below. However, RT-PCR with forward and reverse primers selected to obtain overlapping sequences covering the entire sequence of vasopressin V_2 mRNA gave not only normal products (Birnbaumer et al., 1993), but also one abnormally sized product. The abnormal form was obtained as a second product when primers spanning intron 2 were used and was larger by the size of this intron (~100 bp) than the size of 862 bp, predicted from the structure of V_2 receptor mRNA. A similar product was earlier reported by us to be a product of small-cell lung cancer (North et al., 1998). Cloning and sequencing of V_{1a} and V_{1b} related products, and V_2 related products of predicted size, showed them to collectively provide a complete characterization of human V_{1a} mRNA for the MCF-7 breast cancer cell line from -23 at the 5' end (23 bases prior to the reading frame) through 1224 at the 3' end (18 bases beyond the reading frame), for V_{1b} mRNA from 123 bases beyond the 5' end to 52 bases beyond the 3' end, and for V_2 mRNA from 32 bases beyond the 5' end to 53 bases beyond the 3' end. The sequences for the vasopressin V_1 receptor mRNAs had exact sequence homology with the sequence of human V_{1a} mRNA and the sequence of human V_{1b} mRNA published earlier by us and others (North et al., 1998; Thibonnier et al., 1994; Sugimoto et al., 1994; de Keyzer et al., 1994). One sequence of human V_2 receptor mRNA from breast cancer cells was identical to that published by us and others for normal human tissues (Fay et al., 1996; Birnbaumer et al., 1993). Additionally, an enlarged product of the V_2 receptor was found to contain the entire 106 bases of intron 2 in addition to sequence for V_2 mRNA.

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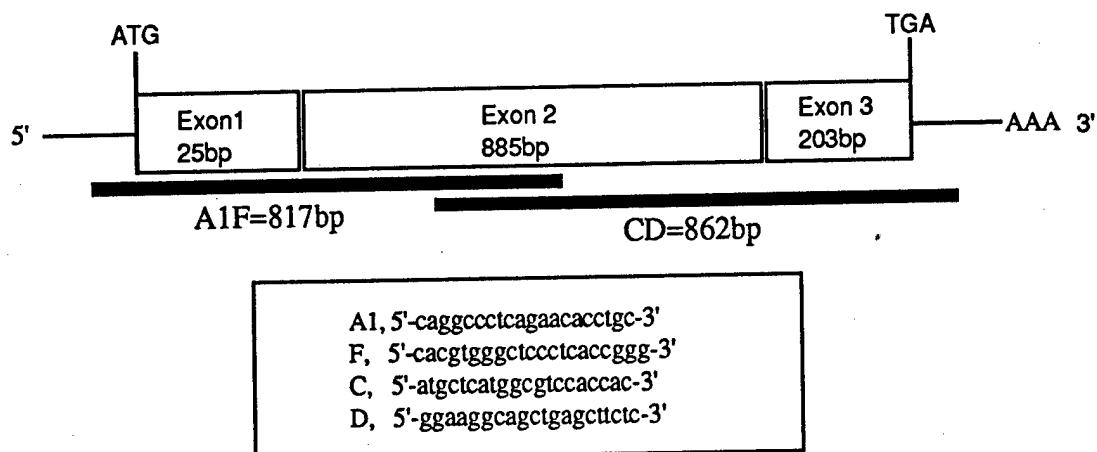
RT-PCR primers and products of V1a receptor in MCF-7 breast cancer cells



RT-PCR primers and products of V1b receptor in MCF-7 breast cancer cells



RT-PCR primers and products of V2 receptor in MCF-7 breast cancer cells



(7) Conclusions

The studies conducted over the four years of the funding period have further confirmed our original hypothesis that all breast cancers produce vasopressin as an autocrine growth factor, and that this property can be utilized to develop more successful treatments. Expression of the vasopressin gene seems to be associated with all oncogenic transformation of breast tissue as evidenced by the presence of vasopressin gene products in all breast cancers examined, the absence of these products from all varieties of fibrocystic disease, and our demonstration that all carcinoma *in situ* (DCIS) examined expressed these same products. We our findings can have short-term clinical application in providing earlier detection of breast cancer as an effective way to distinguish atypical intraductal hyperplasia from carcinoma *in situ*. There is no other simple one-step method available for making this distinction.

When vasopressin gene(s) are expressed by breast cancers, they give rise to normal and abnormal products. Studies conducted by us on trafficking of vasopressin gene-related products by breast cancer cells has revealed that about nine-tenths of the proteins become components of the plasma membrane able to provide targets for antibodies in patients. These proteins comprise both a 40 KDa and 20 KDa form. Antibodies against one of the recognized abnormal structures in these vasopressin gene-related proteins are now being produced. Such new or already available antibodies should be potentially useful in later planned immunodiagnosis and immunotherapies. Alternatively, antibodies already available to normal VP-related structures should also be effective for targeting breast cancer in patients.

Vasopressin seems to have a multifaceted role on the growth and physiology of breast cancer cells because we have demonstrated that all known forms of vasopressin receptor subtypes are expressed by these cells. The complete sequences of a putative receptor named hVACM has been determined by us and entered in the Genebank. Structures for vasopressin V_{1a} , V_{1b} , V_2 , and abnormal V_2 receptors have also been determined. Sequence data shows they have the same sequences submitted to the Genebank by us for vasopressin receptor subtypes produced by small-cell carcinoma cells. We have further demonstrated that through one or more of these receptors, vasopressin is able to alter calcium homeostasis and activated MAPK kinase in breast cancer cells.

Although this represents the final report, we still anticipate continuing a limited number of studies with some carryover funding and these will relate to regulation of vasopressin gene expression and the binding of antibodies to cancer cells *in vitro* and *in vivo*, as designed to satisfy tasks 5 and 6 in the original

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statement of work. We also anticipate publishing more of the above data and will forward such publications as they arise.

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APPENDIX

Publications

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APPENDIX

Report

Vasopressin gene related products are markers of human breast cancer

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Key words: breast carcinoma, immunohistochemical analysis, oxytocin gene expression, tumor markers, vasopressin gene expression

Summary

Immunohistochemical analysis for products of vasopressin and oxytocin gene expression was performed on acetone-fixed tissues from 19 breast cancers representing a variety of tumor sub-types. Studies employed the avidin-biotin complex (ABC) immunohistochemical procedure and utilized rabbit polyclonal antibodies to arginine vasopressin (VP), provasopressin (ProVP), vasopressin-associated human glycopeptide (VAG), oxytocin (OT), oxytocin-associated human neurophysin (OT-HNP), and a mouse monoclonal antibody to vasopressin-associated human neurophysin (VP-HNP). Western Blot analysis was performed on protein extracts of fresh-frozen tissues from 12 additional breast tumors. While VP gene related proteins were not detected in normal breast tissue, immunohistochemistry revealed the presence of VP, ProVP, and VAG in all neoplastic cells for all of the tumor tissues examined. Vasopressin-associated human neurophysin was evident in only one of 19 acetone-fixed tumor preparations. However, Western blot analysis for all 12 fresh-frozen tumor samples showed the presence of two proteins, 42,000 and 20,000 daltons, that were immunoreactive with antibodies to VP, VP-HNP, and VAG. Oxytocin and OT-HNP, by immunohistochemistry, were found to be common to cells of normal breast tissues. For tumors, positive staining for OT was observed in 8 of 18 tumors, while OT-HNP was not detected in any of the tumors examined. These findings indicate that VP gene expression is a selective feature of all breast cancers, and that products of this expression might therefore be useful as markers for early detection of this disease and as possible targets for immunotherapy.

Introduction

Breast cancer is the leading cause of death among American women between the ages of 40 and 55 years. It has been predicted that 12% of women, during their lifetime, will be diagnosed as having this cancer, and that 3.5% will die of the disease [1]. Efforts are therefore currently under way to develop improved methods for the earlier detection of breast cancer, and more effective therapeutic interventions [2-4]. It is likely that both of these aims

could be aided by the identification of easily detectable markers of this disease. One or more vasopressin gene related products (VP, VP-HNP, VAG, and ProVP) have been shown to be effective markers for most, or all, small-cell tumors of the lung [5, 6]. Since two of these substances are released from small-cell tumors into the circulation, inappropriately high levels in the plasma of most patients can be employed to evaluate treatment, and to serve as predictors of recurrent disease [5, 7]. As well as this, VP gene expression by small-cell carcinoma of the

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lung (SCCL) leads to the formation of Neurophysin-Related Cell-Surface Antigen (NRSA), a component of the plasma membrane of these tumors that can be targeted in patients with antibodies to neurophysin [8, 9]. Oxytocin gene related products have also been identified in many SCCL tumors. With respect to breast cancer, OT is known to be involved in the physiology of human mammary glands, and VP has been reported to act as a growth factor for some mammary tumor cells in culture [10]. Therefore, in this study, antibodies to different VP and OT gene-related products were employed for the immunohistochemical screening of an archival library of breast tumors made available through the Department of Pathology at the Dartmouth-Hitchcock Medical Center. In addition, Western blot analysis was performed on fresh-frozen tumor samples with three of the antibody preparations.

Materials and methods

Tissue specimens

Fresh samples of breast tumor were obtained by surgical resection from patients treated at the Dartmouth-Hitchcock Medical Center. These tissue samples represented a range of tumor histological types [11]. Of the 19 tumor samples used in the study, 12 were obtained through biopsy of the breast, 5 were from mastectomies, and 2 were from metastatic sites. A tissue sample of normal human cerebral cortex, and tissue samples of normal human pituitary and hypothalamus, were obtained from autopsy, and these served as negative and positive controls for immunohistochemical staining. All of the tissues were fixed with acetone using the AMeX procedure [12], and then embedded in paraffin. Fresh-frozen tissues from 12 additional breast tumors used in Western blot analysis were obtained on dry-ice from the Cooperative Human Tissue Network, Philadelphia, PA.

Polyclonal antibodies

Rabbit polyclonal antibodies to VP, to OT, and to a synthetic C-terminal 18 amino acid residue of vasoressin-associated human glucopeptide (VAG₂₂₋₃₉) were all prepared here by employing the purified substances, coupled to thyroglobulin with glutaraldehyde, as antigens. Antibodies to VP (Gonzo 3) have been successfully employed in RIA at a dilution of 1:80,000, and in this assay showed < 0.1% cross-reaction with OT and < 0.1% cross-reaction with vasotocin. Hence, Gonzo 3 would seem to contain VP ring directed antibodies as dominant constituents. Antibodies to OT (Kermit 5) were effective in RIA at 1:50,000, and displayed < 0.2% cross-reaction with VP and < 1% cross-reaction to vasotocin. This preparation is, therefore, believed to comprise chiefly OT tail-directed antibodies. The OT-HNP antibody preparation (Archie 3) has been described previously [13], and seems to contain dominant antibodies directed against the unique N-terminal region plus one of the conserved central sequences of this protein. The first three were used in immunohistochemical studies at dilutions of from 1:200 to 1:400; VAG antibodies (Boris Y2) were used at dilutions of from 1:600 to 1:1000.

Rabbit polyclonal antibodies (YL3) that recognize the tripeptide bridge connecting VP to VP-HNP in ProVP were a generous gift from Joseph Verbalis of Pittsburgh, PA. Dr. Verbalis employed a synthetic dodecapeptide that represents the tripeptide tail of VP, the tripeptide bridge, and the first six amino acid residues of VP-HNP as antigen for raising these antibodies. These antibodies were shown [14] to uniquely react with ProVP, and not with VP or VP-HNP. In the present studies they were used at serum dilutions of from 1:200 to 1:600.

Monoclonal antibodies

A monoclonal antibody (Nab1) to VP-HNP, that was generated through hybridoma technology by one of us (WGN), was also utilized in immunohistochemical studies and Western Blot Analysis. The antibody was obtained from mouse ascites by affinity chromatography on a column of VP-HNP-Sepha-

rose [9], and used at concentrations from 0.025 to 0.05 μ g/ml. This antibody showed < 1% cross-reaction with OT-HNP in RIA.

Immunohistochemical staining

Tissue sections of 4 μ m were examined using the avidin-biotin complex (ABC) immunohistochemical procedure (Vector Laboratories, Burlingame, CA). Following the removal of paraffin by washing in xylene (3 \times 5 min) and acetone (30 sec), tissue sections were rinsed with PBS (2 \times 3 min) and incubated with 1.5% goat serum (for studies with rabbit antibodies) or 1.5% horse serum (for studies with the mouse monoclonal antibody), supplied by Vector Laboratories, at ambient temperature to block non-specific binding. After removal of blocking serum by aspiration, tissues were incubated with the relevant primary antibody diluted with 10% goat serum (polyclonal antibodies), or 10% horse serum (monoclonal antibody), at 4°C for 24 hr. Primary antibody was removed by rinsing in PBS (2 \times 3 min), and biotinylated secondary antibody, comprising either goat anti-rabbit IgG or horse anti-mouse IgG, applied at a concentration of 20 μ g/ml for 30 min at ambient temperature. Following the removal of unbound secondary antibody with PBS (2 \times 3 min), endogenous peroxidase was blocked with a methanol solution containing 3% hydrogen peroxide [15]. Tissues were then rinsed with PBS and incubated with an avidin-peroxidase complex (25 μ g/ml) for 30 min at ambient temperature. Excess complex was removed with PBS (2 \times 3 min), and the bound complex was visualized by the peroxidase oxidation of 3,3'-diaminobenzidine over 3 min when presented as a 0.2 mg/ml solution with 0.03% hydrogen peroxide. Counterstaining was performed with hematoxylin. Tissues were then dehydrated through increasing concentrations of ethanol to xylene and coverslipped with permount. In order to record findings with black and white photography, this counterstaining was later removed with acid-alcohol (0.8 M HCl in 95% ethanol). Lack of staining in sections incubated with normal serum instead of primary antibody preparations was used to demonstrate antibody specificity.

Western Blot analysis

Protein from finely minced tumor tissue was extracted by sonication in 100 volumes of 0.1 M HCl. Suspensions were centrifuged at 1500 \times g for 10 min at ambient temperature, and soluble protein was then precipitated with 40% TCA. This protein was pelleted by centrifugation at 10,000 \times g for 2 min. TCA was then removed from pellets by washing (x 2) with ether. Protein was resuspended in 0.1 M Tris HCl (pH 8.7), reduced with mercaptoethanol at 100°C for 5 min (and in some cases S-alkylated with N-ethyl maleimide), and subjected to SDS-PAGE electrophoresis on 15% gels at pH 9.3 using the method of Laemeli [16]. Separated proteins were then electrophoretically transferred with 20 mM Tris glycine (pH 8.0) to Immobilon (PVDF) membranes. These membranes were blocked with a 5% non-fat milk solution, washed (1 \times 15 min, 2 \times 5 min) with PBS containing 0.5% Triton, and reacted with preparations of mouse monoclonal antibody to VP-HNP, with rabbit polyclonal antibodies to VP, with rabbit polyclonal antibodies to VAG, or with ubiquitous mouse or rabbit IgG (negative controls), for 1 h at ambient temperature. Following a second wash in PBS-Triton (1 \times 15 min, 2 \times 5 min), the membranes were treated, respectively, with goat anti-mouse IgG-horseradish peroxidase conjugate or goat anti-rabbit IgG-horseradish peroxidase conjugate for 1 h, and then washed with PBS-Triton (1 \times 15 min, 4 \times 5 min). Immunoreactive proteins were visualized using an ECL Western Blotting Detection System with exposure of x-ray film from 10 seconds to 5 min. Prestained SDS-PAGE standard proteins were employed as molecular size markers.

Results

Immunostaining with antibodies to VP, ProVP, and VAG

All tumor cells, for all tumor specimens examined, showed intense positive immunological staining with antibodies to VP, and to the C-terminal region of VAG. Positive immunostaining with antibodies to the bridging region of ProVP was found in 11 of 14



Fig. 1. Acetone-fixed breast carcinoma showing colloid-type inclusions. Positive staining with rabbit polyclonal antibodies to vasopressin, using the Avidin-biotin complex immunohistochemical procedure and peroxidase oxidation of 3,3'-diaminobenzidine (magnification, $\times 1500$).

tumors tested. Tumor tissues represented all forms that typify breast carcinoma (Table 1). Figure 1 represents a colloid carcinoma and immunostaining obtained with antibodies to VP. Figure 2 is the immunostaining that typified the reaction of infiltrating lobular carcinoma with antibodies to VAG. No immunostaining of the normal connective and glandular tissues surrounding tumors, or with cerebral cortex, was obtained with antibodies to VP, ProVP, or VAG.

Immunostaining with antibodies to OT and VP-HNP

Some tumor tissues (8 of 18 tested), as well as surrounding normal glandular tissue, stained with polyclonal antibodies to OT. Oxytocin-associated human neurophysin was not detected in any of the 19 tumors examined. The mouse monoclonal antibody to VP-HNP failed to react with all but one of the 19 tumor specimens (an infiltrating lobular carcinoma) and gave no immunostaining of surrounding normal tissues.

Western blot analysis with antibodies to VP and VP-HNP and VAG

Both polyclonal antibodies to VP and VAG and the mouse monoclonal antibody to VP-HNP gave similar patterns of immunoreactive proteins with electrophoreograms from Western blot analysis of 12 fresh-frozen tissues of breast cancer. In each case, immunoreactive proteins were discerned with apparent molecular weights of 40,000 and 20,000 daltons. In addition to these, the antibody to VP-HNP reacted weakly with a protein of approximately 10,000 daltons. The pattern obtained for four tumor samples with the antibody to VP-HNP is represented in Fig. 3.

Discussion

The results obtained in this study indicate that the VP gene is expressed by all breast cancers, and that VP is a product common to all these tumors while being immunologically absent from surrounding normal breast tissue. This tumor VP could be per-

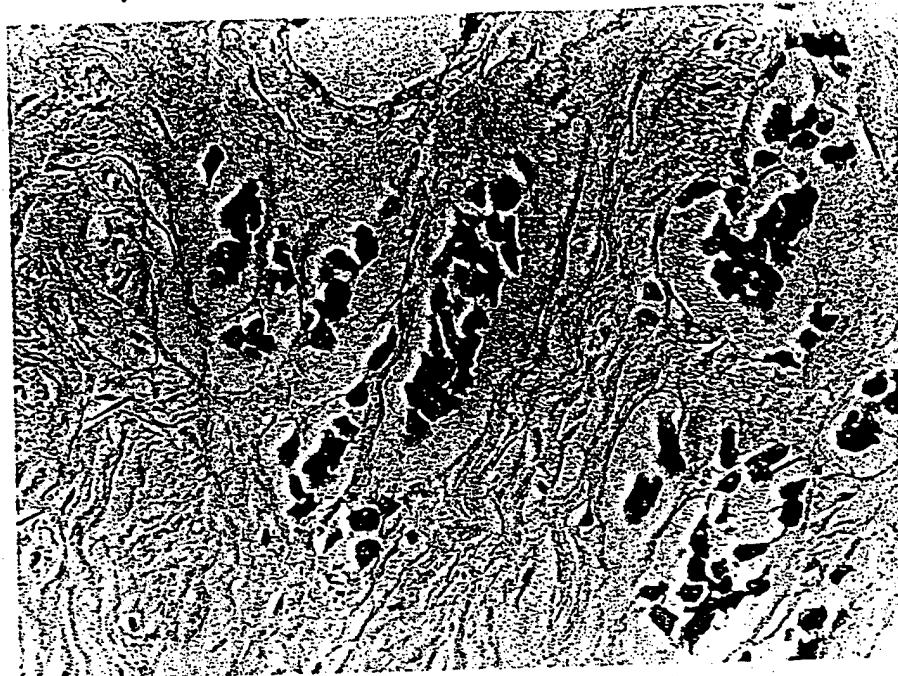


Fig. 2. Acetone-fixed breast carcinoma of the infiltrating lobular sub-type showing positive immunostaining with rabbit polyclonal antibodies to vasopressin-associated human glycopeptide. The procedure employed was avidin-biotin complex immunohistochemistry with peroxidase oxidation of 3,3'-diaminobenzidine (magnification, $\times 480$).

Table 1. Presence of vasopressin and oxytocin gene related products in human breast cancer

Cancer subtype	VP gene related antigens*				OT gene related antigens*	
	VP	VAG	VPRG	ProVP	OT	OT-HNP
Infiltrating ductal	na	-	+	na	na	-
Infiltrating ductal	+	-	+	+	-	-
Infiltrating ductal	+	-	+	+	+	-
Infiltrating ductal	+	+	+	+	+	-
Infiltrating ductal	+	-	+	+	-	-
Infiltrating ductal	+	-	+	-	-	-
Infiltrating ductal	+	-	+	+	+	-
Infiltrating ductal	+	-	+	na	+	-
Infiltrating ductal	+	-	+	+	-	-
Infiltrating ductal	+	-	+	+	-	-
Infiltrating ductal	+	-	+	+	-	-
Infiltrating ductal	+	-	+	-	-	-
Infiltrating ductal	+	-	+	-	+	-
Infiltrating ductal	+	-	+	-	-	-
Colloid	+	-	+	+	-	-
Colloid	+	-	+	na	-	-
Colloid	+	-	+	+	+	-
Colloid	+	-	+	+	+	-
Infiltrating tubular	+	-	+	na	+	-
Infiltrating tubular	+	-	+	+	-	-
Infiltrating lobular	+	-	+	na	-	-
Total positive	18/18	1/19	19/19	11/14	8/18	0/19

* Positive (+) or negative (-) immunoreactivity using antibody preparations and the ABC procedure. na = not attempted.

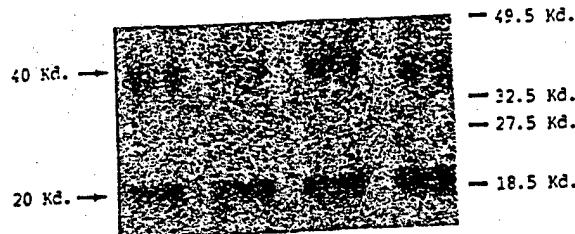


Fig. 3. Western blot analysis from SDS-PAGE (15%) showing mercaptoethanol-reduced proteins from four biopsied human breast tumors that were immunoreactive with a mouse monoclonal antibody (NAB 1) to vasopressin-associated human neuropephsin. The PVDF membrane was blocked with non-fat milk as a 5% solution in PBS-Triton. Incubations with 0.5 µg/ml primary antibody, and then with goat anti-mouse IgG-horseradish peroxidase conjugate (BRL), in 5% nonfat milk, were each performed for 1 h at room temperature. Immunoreactive proteins were visualized on x-ray film exposed to the membrane for 5 min.

forming a role as an autocrine growth factor in the same manner as it appears to do for many small-cell tumors of the lung [17]. Taylor and coworkers [10] have shown that VP may be an important growth modulator of human breast cancer, and other workers [18] have described a growth-promoting action of the peptide on rat mammary tumors in culture.

While the origin of human breast cancer is not yet clear, an increased risk is associated with atypical hyperplasia, especially of both ductal and lobular types. It is possible that some hyperplasia conditions become malignant as a consequent of an over-production of growth factors by the affected cells. Since VP was found to be common to all of the breast tumors examined in this study, it is conceivable that this peptide is also a marker of those proliferating cells that serve as progenitors of breast cancer. Such a hypothesis is readily testable and, if true, might lead to a method for recognizing pre-cancerous cells in patient biopsies.

Vasopressin-associated human glycopeptide was another marker immunologically identified in all of the breast cancers examined. This secretory product of hypothalamic neurons has yet to have a defined physiological function. However, VAG has been demonstrated to be a component of NRSA in SCCL, and as such is a possible target on the plasma membrane of these cells for radiodiagnostic imaging and immunotherapy [9]. If this entity, as a com-

ponent of proteins, also becomes incorporated into the plasma membranes of breast cancer cells, it would then seem to be an ideal candidate through which imaging and treatment of this disease could be carried out. In support of this possibility are the findings presented here from Western Blot analysis of VAG immunoreactivity, that demonstrate it to be associated with a larger protein(s) of the same molecular size as those found to occur in SCCL [8]. These are proteins of 40,000 and 20,000 daltons that also react with antibodies to VP and VP-HNP. Nevertheless, differences between the products of breast cancer cells and small-cell tumors are indicated by the failure of our monoclonal antibody to VP-HNP to positively stain all but one of the breast cancer specimens, even though this antibody reacted with breast cancer proteins in Western blot preparations. This suggests there might be even more conformational abnormalities in breast cancer-produced neuropephsin structures than occur in small-cell carcinoma, and this in turn, might reflect more significant substitutions and/or omissions in the exon B and exon C regions of the expressed VP gene of breast cancer cells. Staining of similar intensity with VP antibodies and antibodies to the peptide bridging region of ProVP for most tumors makes it less likely that significant abnormalities are present in the exon A region of the VP gene(s) expressed by breast cancer cells [19].

Oxytocin, in contrast to VP gene related products, appeared to be common to both normal breast tissue and some tumor cells. Staining for OT was evident in 2 of 4 tumors of the colloid sub-type, in 5 of 12 ductal tumors, and in 1 out of 2 of the tubular sub-type, but was not evident in the one lobular tumor examined. The data indicated that overall, the OT gene is expressed in about 44% of breast carcinomas, although there is evidence that gene expression may be abnormal because none of the tumors appeared to contain a structure immunologically identified as OT-HNP.

Hence, only VP gene expression would seem to be a universal feature of breast carcinoma. While there is evidence that this VP gene gives rise to abnormal protein products, these products are always recognized in immunological studies by antibodies to VP and VAG. These substances, would therefore

appear to represent important markers of this disease and, as such, could be effectively utilized in its diagnosis and treatment.

Acknowledgement

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Factors Regulating the Production of Vasopressin-Associated Human Neurophysin by Small-Cell Carcinoma of the Lung: Evaluation by Computer-enhanced Quantitative Immunocytochemistry

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Abstract—Expression of the vasopressin gene appears to be a property common to all small-cell lung tumours. For some cultures of small-cell lung carcinoma (SCCL), Northern and Western Blot analyses have revealed that expression of this gene and its protein products are regulated by cAMP and glucocorticoids. In this study, these evaluations have been extended by examining the production of vasopressin-associated human neurophysin (VP-HNP) by computer-enhanced quantitative immunocytochemistry in a classical cell-line (H69) of SCCL, and defining the amount of protein in cells by area of positive staining above an arbitrarily set threshold. Intracellular cAMP was raised by incubating cells with either 8-Br-cAMP (0.5 mM) and IBMX (0.5 mM), or with forskolin (25 μ M) and IBMX (0.5 mM). Both of these treatments caused a significant increase in the amount of positive VP-HNP immunoreactivity in the cells, an increase that was further enhanced by simultaneous administration of dexamethasone (0.1 μ M). Addition of dexamethasone alone, however, caused a significant decrease in VP-HNP levels. Results confirm earlier findings from Western Blot analysis revealing the influence these agents have on production of vasopressin gene-related proteins by H69 cells, and indicate that computer-enhanced quantitative immunocytochemistry can be effectively used to provide a suitable index of this production.

Introduction

The physiological parameters that regulate the expression of vasopressin (VP) in the central nervous system are dependent upon cell type and locale. Perhaps the most widely recognized stimuli

for VP production are increases in osmolality and reduction in blood volume. These changes are known to increase synthesis by magnocellular neurons in the hypothalamus,¹⁻³ through processes thought to involve cAMP.⁴⁻⁸ In the parvocellular subdivision of the paraventricular nucleus, levels of VP-mRNA are reduced by glucocorticoids,⁹⁻¹² while neurons in the suprachiasmatic nucleus vary their expression of VP depending upon the time of day.¹³⁻¹⁷ In extrahypothalamic regions of the brain, androgens inhibit peptide production by vaso-

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pressinergic cells of the bed nucleus of the stria terminalis.^{18,19}

In contrast to the well-regulated expression of VP by neurons of the central nervous system, expression of the peptide by small-cell carcinoma of the lung (SCCL) has long been thought to be largely autonomous.²⁰ SCCL production also involves the generation of unusual forms of vaso-pressin-associated human neurophysin (VP-HNP), that become incorporated into the cell membrane as NRSA (Neurophysin-Related Cell Surface Antigen) instead of being secreted. However, Verbeeck and co-workers were able to show for one classical SCCL cell line that VP mRNA levels are influenced by cAMP and glucocorticoids.²¹ Additionally, in this laboratory, these agents were shown to also influence the production of VP-HNP and NRSA by four different cell lines of classical SCCL.^{22,23} In this study, affinity purified antibodies were employed in conjunction with computer enhanced imaging to confirm that classical SCCL has the capacity to regulate VP gene expression.

Materials and methods

Cell culture

SCCL H69 cells (American Type Culture Collection, Rockville, MD, USA) were grown in 75 cm² tissue-culture flasks containing 30 ml of RPMI medium (JRH Biosciences, Lenexa KS, USA) with 10% fetal bovine serum and gentamicin (50 µg/ml, United States Biochemical Corporation, Cleveland, OH, USA). In experiment A, cells were treated with either a combination of isobutyl-methylxanthine (IBMX, 0.5 mM) and forskolin (25 µM, Sigma, St Louis, MO, USA) or vehicle (0.1% ethanol). For experiment B, cells were divided into four groups: the first group was incubated with IBMX (0.5 mM) and 8,bromo-cAMP (0.5 mM, Boehringer Mannheim, Germany); the second group with dexamethasone (0.1 µM, Sigma); the third group with IBMX (0.5 mM), 8,bromo-cAMP (0.5 mM), and dexamethasone (0.1 µM); and the fourth group with vehicle as a control. Incubation with these reagents was carried out for periods of 2–8 d. Media, containing the appropriate reagent(s), was changed every 48 h. Two days prior to immunocytochemical staining, cells were transferred to a Lab-Tek tissue

culture-chamber/slide (Miles Scientific, Naperville, IL, USA), containing 8 × 1 cm² wells.

Immunocytochemistry

Following removal of the culture medium, cells were fixed by immersion in acetone for 2 min. The fixative was removed by two 3-min washes with phosphate-buffered saline (PBS).

The avidin-biotin complex (ABC) immunohistochemical procedure used in this study was based on that by Guesdon et al²⁴ and its use in our laboratory has been more fully described in earlier publications.^{25,26} All incubations were carried out at room temperature and cells were not pretreated with a hydrogen peroxide and methanol solution because H69 cells do not display any endogenous peroxide activity in the ABC immunocytochemical procedure. The primary antibody solution consisted of a rabbit polyclonal preparation, Ralph-4(88), that was directed against VP-HNP. Biotinylated anti-rabbit goat serum was employed as the secondary antibody solution. To improve specificity and reduce background, the primary antibodies were purified on a column containing Sepharose-bound, purified, VP-HNP.²⁷ Prior to use, the purified antibodies were diluted to a concentration of 27 µg/ml. Antibody specificity was further determined by the positive identification of magnocellular neurons in paraffin-embedded sections of hypothalamus, a lack of staining in H69 cells when normal goat serum was substituted for primary antibody, and the inability of antibody to label cultured fibroblast cells. Following the visualization step with 3,3' diaminobenzidine, cells were dehydrated in ascending ethanol to xylene and coverslipped.

Image analysis

The area of staining for VP-HNP was quantitated using a microcomputer imaging device (MCID, Imaging Research, Inc., Toronto, Canada). An Olympus BH-2 microscope equipped with a 10× objective, and a Sierra Solid State camera were used to project the field view onto a RGB Electrohome Video Monitor. Computer analysis of this monitor determined the optical density of the screen pixels, represented by a scale of 256 gray levels. Two optical density thresholds were selected prior to

quantitation, a lower value that comprised a background of total cell area and a higher one that represented positive immunostaining. The same optical thresholds were used throughout the analyses for each experiment. Using these thresholds, the computer scanned the cells displayed on the monitor to determine, in pixels, the total cell area (A_T) and the area stained for VP-HNP (A_S) (Fig. 1A & 1B). The area of immunoreactivity, or percentage of cell area staining for VP-HNP ($\%A_S$), was calculated using the following formula: $\%A_S = 100 \times A_S/A_T$. To ensure adequate and non-biased sampling, each treatment group was analyzed using a grid containing 1 mm² squares. The grid was aligned over the borders of each slide-well examined, and the $\%A_S$ was determined only in grid squares containing a total cell area of greater than 1500 pixels (representing approximately 15–20 cells). This technique resulted in sampling an average of 26 (± 4 SEM) grid squares per treatment group. The mean $\%A_S$ for each treatment group was calculated from these values. Statistical significance ($p < 0.05$) was determined using an unpaired Student's two-tailed t-test for experiment A and analysis of variance (ANOVA) for experiment B. A significant ANOVA was followed by a Fisher's post-hoc comparison.

Results

Neurophysin immunoreactivity

H69 cells displayed a heterogeneous pattern of VP-HNP staining, with some cells strongly immunoreactive and others only slightly so. As was found earlier for neurophysin immunoreactivity of the SCCL cell line DMS-240,²⁸ the majority of staining in many cells, both treated and untreated, was localized to one side of the cell and was closely associated with the plasma membrane (Fig. 1B). This localized staining was clearly undiminished by any of the treatments, although it could not be quantitated. Staining was not observed in H69 cells when normal goat serum was substituted for primary antibody, or in cultured fibroblast cells incubated with the same concentration of Ralph-4(88) that labelled H69 cells.

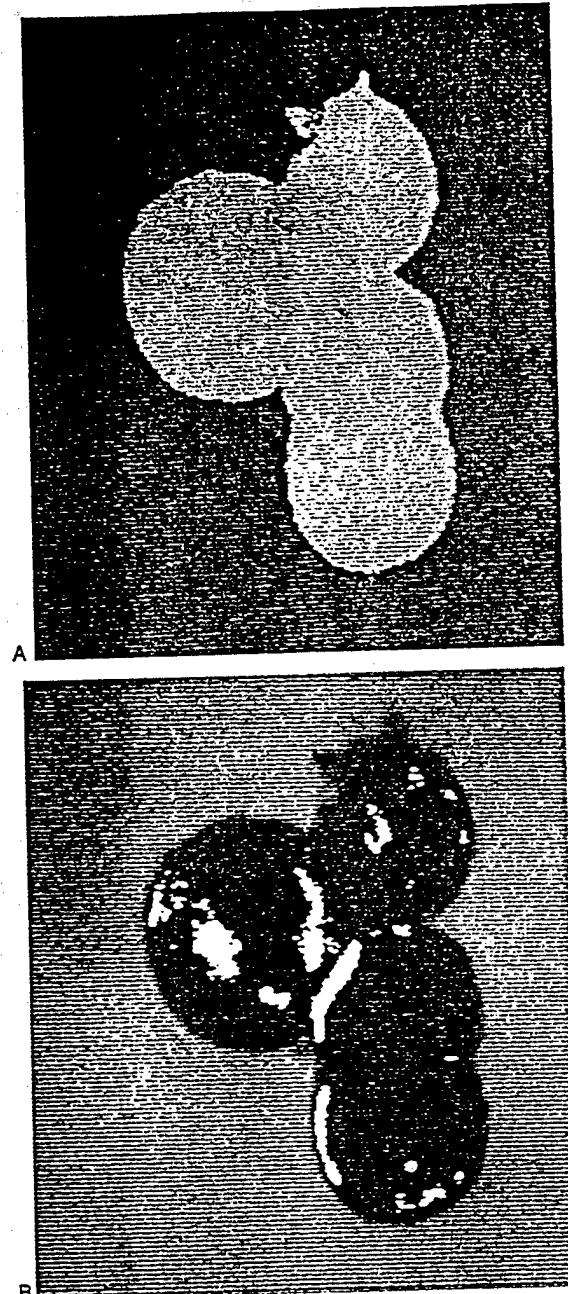


Fig. 1 (A) The total cell area (A_T), represented in white, of a group of H69 cells on the RGB Electrohome Video Monitor, comprising all pixels with a density above background. (B) The area stained for VP-HNP (A_S), also represented in white, of the same group of H69 cells shown in (A).

Experiment A

The results from experiment A are summarized in Figure 2. After 2 days of treatment with IBMX and forskolin, the percentage of cell area showing

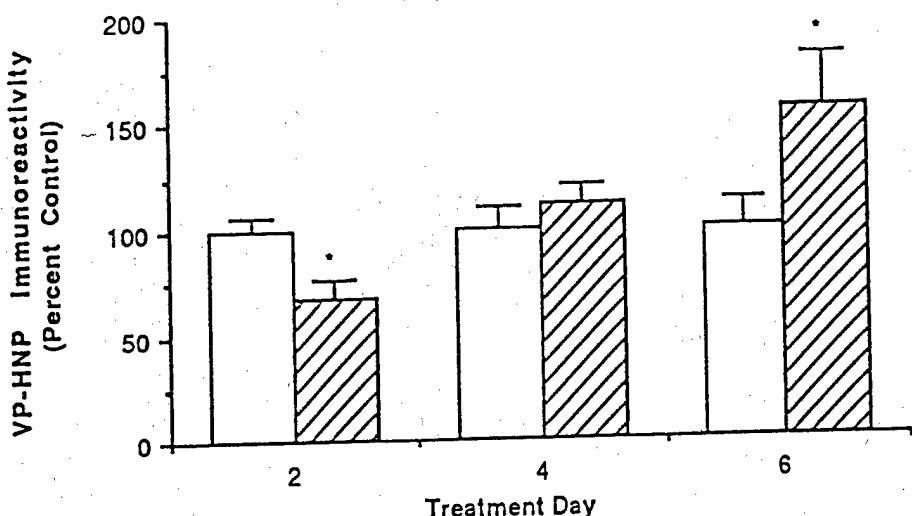


Fig. 2. Distribution of VP-HNP immunoreactivity in H69 cells after treatment with vehicle (open bars), or with IBMX and forskolin (closed bars). Values are expressed as percent control and error bars represent \pm SEM. * Indicates a significant difference from control.

immunoreactivity for VP-HNP was less than that in the cells receiving vehicle. Although not significant, by day 4 of treatment this change appeared to be reversed, with an observed marginal increase in the area of VP-HNP immunoreactivity over that of vehicle. By day 6 of treatment, the area stained for VP-HNP was roughly 1.5 times that in the control group.

Experiment B

The effects of treating H69 cells with different combinations of IBMX, 8,bromo-cAMP, and dexamethasone on VP-HNP immunoreactivity in H69 cells are summarized in Figure 3A, B and C. On day 2 of treatment, cells incubated with IBMX plus 8,bromo-cAMP displayed a greater area of VP-HNP immunoreactivity than control cells, while treatments with dexamethasone alone, or with a combination of dexamethasone, IBMX and 8,bromo-cAMP, significantly decreased the area of VP-HNP staining (Fig. 3A). After 4 days of treatment, the effects obtained were the same as those after 2 days, with the notable exception that cells exposed to all three drugs had a larger percentage area staining for VP-HNP than the cells in both the control group and the group treated with IBMX and 8,Br-cAMP (Fig. 3B). By day 8 of treatment, any influence of these reagents on the area of neurophysin staining was no longer detectable, with

analysis of variance revealing no significant differences among all of the groups examined (Fig. 3C).

Discussion

Cyclic AMP-dependent processes (mediated by protein kinase A) and glucocorticoids have been implicated in the regulation of hypothalamic VP gene expression.⁶⁻¹² The results of this study indicate these factors can also influence VP production by classical SCCL. Since treatment did not diminish staining at the plasma membrane of cells, and since there is very minimal release of protein by these cells, an increase in the area of staining is concluded to represent an increase in VP-HNP production. This conclusion is supported by Western Blot analysis and cytofluoremetry performed on H69 cells.^{22,23}

In the first experiment, we sought to indirectly stimulate protein kinase A in H69 cells by activating endogenous adenyl cyclase with forskolin and inhibiting phosphodiesterase with IBMX. After 6 days, this treatment caused an increased distribution of immunoreactivity for VP-HNP (Fig. 2). Direct stimulation of protein kinase A with 8,Br-cAMP, in the presence of IBMX, confirmed our initial finding indicating that protein kinase A activation increases VP-HNP immunoreactivity in H69

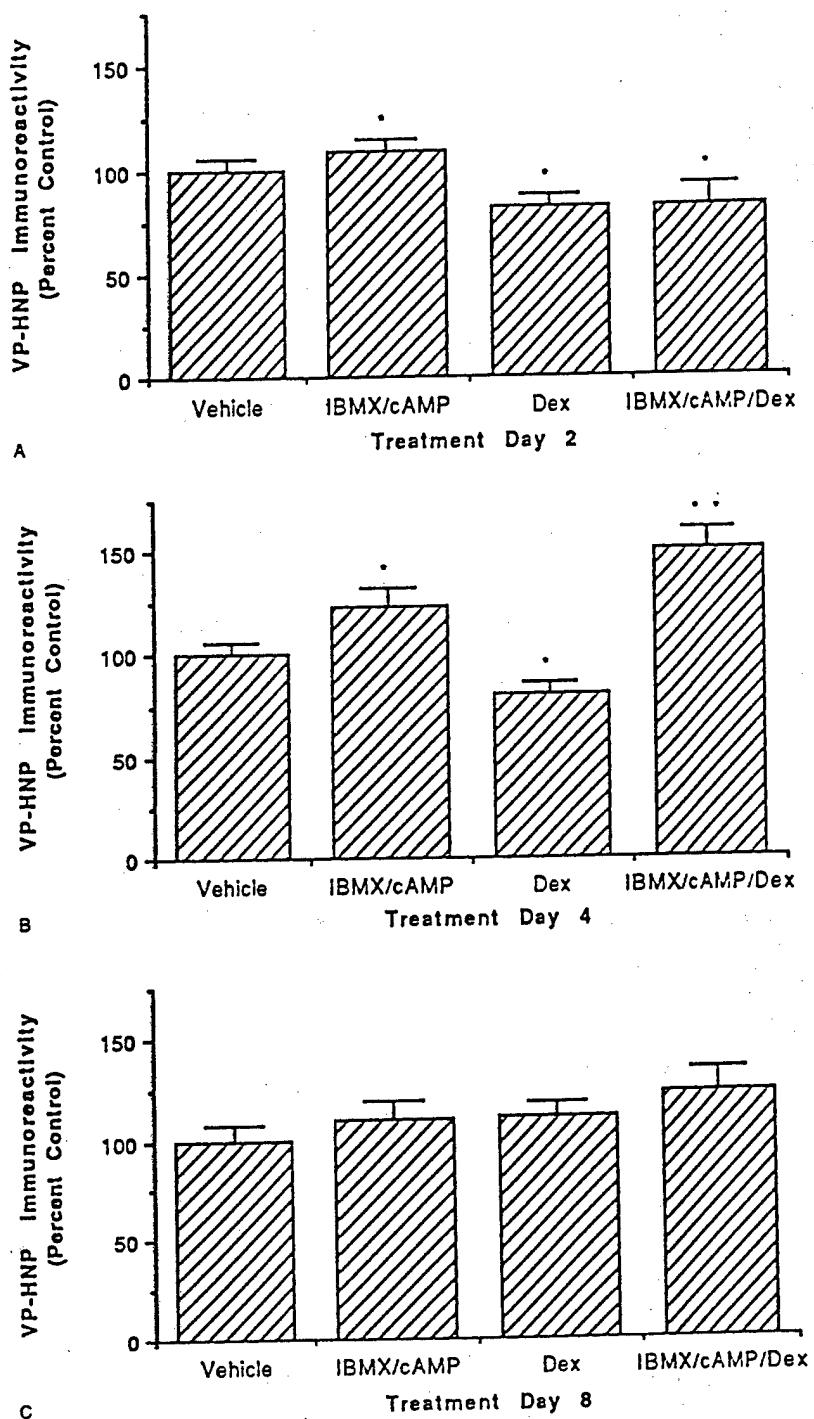


Fig. 3 Distribution of VP-HNP immunoreactivity in H69 cells treated with combinations of IBMX, 8-bromo-cAMP, and dexamethasone after 2 (Fig. 3A), 4 (Fig. 3B), and 8 (Fig. 4C) days. Values of $\%A_5$ were compared using ANOVA and are expressed relative to control. * Represents a significant difference when compared to control, and ** in Figure 3B indicates a significant difference from immunoreactivity in cells treated with IBMX, 8-bromo-cAMP or vehicle. Error bars represent \pm SEM.

cells. On days 2 and 4 of treatment, cells cultured with 8,Br-cAMP and IBMX increased production of neurophysin (Figs 3A & B). Unlike indirect stimulation of protein kinase A with IBMX and forskolin, cells treated with 8,Br-cAMP and IBMX did not display a transient decrease in neurophysin production on the second day of treatment. This transient decrease might therefore have been due to an early, independent effect of forskolin.

Supporting the finding that VP-HNP synthesis is increased by these drugs are results from a number of studies that have revealed stimulation of intracellular cAMP levels produces an enhanced expression of the VP gene. Young and co-workers⁶ reported that dehydration caused an increase in both hypothalamic VP-mRNA and the mRNAs for the G-proteins that regulate adenylyl cyclase activity, and others have demonstrated a positive correlation between hypothalamic cAMP levels and VP-mRNA during osmotic stress.^{7,8} More particularly related to the present study, Verbeeck et al, working with the classical SCCL cell line GLC-8, found that incubation for 2 days with 8,Br-cAMP and IBMX doubled the level of VP-mRNA.²⁹ Moreover, Western analysis has demonstrated that the intracellular levels of VP-HNP are increased by 8,Br-cAMP and IBMX for the cell line studied here (H69) and for additional cell lines of classical SCCL.^{22,23}

Other actions of forskolin, IBMX, and 8,Br-cAMP must also be considered when interpreting their effects on VP-HNP immunoreactivity in H69 cells. It has been suggested, for instance, that forskolin, even at concentrations lower than that used in our study, can indirectly stimulate protein kinase C.³⁰ Interestingly, Verbeeck et al²¹ found that stimulation of protein kinase C with phorbol esters increased VP-mRNA in SCCL cells, although subsequent work by others did not reveal such a response in cultures of hypothalamic neurons.³¹

Expression of the VP gene in parvocellular neurons of the paraventricular nucleus is thought to be suppressed by circulating levels of glucocorticoids.⁹⁻¹² In rats, adrenalectomy greatly enhances the level of VP-mRNA in these neurons, an effect that is reversed by administration of dexamethasone.⁹ Our current finding that dexamethasone appears to reduce VP-HNP production in H69 cells suggests that glucocorticoids also have the ability to down-regulate basal expression of VP

by SCCL. The detection of glucocorticoid receptors in a SCCL cell line by others supports this possibility.²¹ Interestingly, when H69 cells have been stimulated to produce greater amounts of VP for 4 d, dexamethasone appears to act synergistically with 8,Br-cAMP and IBMX to further enhance production (Fig. 3B). These seemingly paradoxical influences that glucocorticoids have on VP-HNP production are similar to those reported for VP-mRNA levels,²¹ with one notable exception. While the synergism between dexamethasone and 8,Br-cAMP and IBMX was reflected in mRNA levels within 2 d of treatment, we did not observe effects on VP production until the fourth day. On the second day of treatment, both dexamethasone alone, and dexamethasone in combination with 8,Br-cAMP and IBMX, appeared to decrease production of VP-HNP by H69 cells.

A dual action of glucocorticoids on VP expression has also been noted for the hypothalamic neurons. While glucocorticoids normally inhibit VP expression in parvocellular neurons, in cases of chronic stress this steroid hormone appears to increase synthesis.³² However, unlike SCCL cells, glucocorticoids and cAMP do not appear to interact during stimulation of hypothalamic VP expression. Schilling et al,³³ working on hypothalamic neurons *in vitro*, were unable to demonstrate any synergism between a glucocorticoid antagonist, RU 38 486, and forskolin in their ability to increase VP-mRNA levels.

Hence, the results of the present study, as well as those of others, indicate that SCCL cells possess mechanisms for modulating VP expression in spite of earlier reports that suggested production of this peptide by SCCL tumors is largely unregulated. The clinical significance of SCCL-generated neurophysins has been demonstrated in imaging studies that employed radiolabelled antibodies directed against VP-HNP to target SCCL tumors *in vivo*.²⁷ If neurophysin production by SCCL tumors could be upregulated, the tumor selectivity of radiolabelled anti-neurophysins could be enhanced, and thus provide a more sensitive tool for diagnostic evaluation.

Acknowledgements

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Evidence for Expression of Vasopressin V_2 Receptor mRNA in Human Lung

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FAY, M. J., J. DU, X. YU AND W. G. NORTH. Evidence for expression of vasopressin V_2 receptor mRNA in human lung. PEPTIDES 17(3) 477-481, 1996.—Studies using fetal sheep, goats, and guinea pigs indicate that vasopressin may play a role in preparing the fetal lung for the transition from a uterine to an air-breathing environment by slowing lung liquid secretion. The mechanism of vasopressin action is believed to occur through V_2 receptors with subsequent activation of amiloride-sensitive sodium channels. However, the presence of the V_2 receptor in human lung has not yet been documented. In the present study, expression of the vasopressin V_2 receptor in fetal and adult human lung was examined using reverse transcription-polymerase chain reaction (RT-PCR), Northern blot analysis, and DNA sequencing. Using RT-PCR and primer pairs specific for the human V_2 receptor, PCR products of the predicted sizes of 512 and 862 bp were obtained from adult human lung. DNA sequencing of the cloned PCR products revealed exact identity with the published sequence for the V_2 receptor. Northern blot analysis revealed the expression of a ~1.9 kb mRNA in adult human lung as well as in kidney, but not in fetal human lung at 22–24 weeks of gestation. However, using the more sensitive RT-PCR assay the 862-bp product was successfully amplified from human fetal lung, although the data indicate the mRNA for this receptor is expressed in lower levels than in adult human lung or kidney. Using RT-PCR and primers specific for the rat V_2 receptor, a PCR product of the predicted size of 461 bp was amplified from adult rat lung and kidney, despite an earlier report that this receptor mRNA is absent from the lung of this species. The role for the V_2 receptor in adult human lung is unknown at this time, but, as in the human kidney and lungs of fetal sheep, goats, and guinea pigs, this receptor may play a role in fluid balance.

Vasopressin V_2 receptor mRNA	Human lung	RT-PCR	Northern blot
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THE classical roles for the neuropeptide vasopressin are vasoconstriction through activation of V_{1a} receptors, antidiuresis through activation of V_2 receptors, and modulation of ACTH release through activation of V_{1b} receptors. The transduction cascade associated with V_1 receptors involves the activation of a phospholipase, with the subsequent production of inositol 1,4,5-triphosphate, 1,2-diacylglycerol, calcium mobilization and influx, and protein kinase C activation. The V_2 receptor transduction cascade involves the activation of adenylate cyclase, leading to an increase in cAMP, and the activation of protein kinase A. However, dual signaling potential has been reported with activation of the V_2 receptor (41). In addition, a novel calcium-mobilizing vasopressin receptor (VACM-1) has been cloned and characterized from rabbit kidney (12). With the recent cloning of the human V_{1a} (21,36), V_{1b} (35), and V_2 (7) receptors, an extensive evaluation of vasopressin receptor subtype expression is now possible.

Numerous studies have demonstrated a connection between vasopressin and the lung. Immunoreactive vasopressin has been identified in rat lung extracts, although the exact cellular localization was not determined (1). A common feature of small-cell lung cancer is the ectopic production of vasopressin and vasopressin-related gene products (16,17,25). Also, some small-cell lung cancer cells possess functional V_1 receptors (16,33), which

when activated can stimulate the clonal growth of these cells (33). Also, pneumadin, a decapeptide isolated from lung tissue, has been reported to cause the release of vasopressin from the neurohypophysis (5). In primary rat alveolar type II pneumocytes, vasopressin has been reported to stimulate surfactant secretion through activation of V_{1a} receptors (10,11). Physiological studies indicate that in fetal sheep, goats, and guinea pigs, vasopressin slows lung liquid secretion and, in some cases, causes reabsorption (18,28–30,32,38). These effects of vasopressin were attributed, at least in part, to the action of vasopressin at putative V_2 receptors, with the subsequent activation of amiloride-sensitive sodium channels (13,30,34). However, the expression of this receptor in lung tissue has not yet been demonstrated.

In the present study we used RT-PCR, DNA sequencing, and Northern blot analysis to determine if the vasopressin V_2 receptor is expressed in fetal and adult human lung, and adult rat lung.

METHOD

Tissue Specimens and RNA Isolation

Normal adult human lung tissue specimens were obtained from the Cooperative Human Tissue Network (Philadelphia, PA). Normal adult human lung, fetal human lung, adult human kidney, adult rat lung, and adult rat kidney poly(A)⁺ RNA were

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obtained from Clontech Laboratories, Inc. (Palo Alto, CA). Total RNA was isolated from human lung tissue using TRIzol[®] reagent (Gibco BRL, Gaithersburg, MD). Selection for poly(A)⁺ RNA was performed by oligo(dT) cellulose (Boehringer Mannheim, Indianapolis, IN) chromatography as previously described (3,26).

RT-PCR Analyses

First strand cDNA synthesis was performed using SuperScript[®] II RNase H- reverse transcriptase (Gibco BRL, Gaithersburg, MD) and oligo(dT) priming of the poly(A)⁺ RNA (2–4 μ g). PCR was performed with AmpliTaq[®] DNA polymerase (~2.5 units) and the Gene Amp[®] Kit (Perkin Elmer, Foster City, CA) using the following oligonucleotide primers synthesized by Keystone Laboratories, Inc. (Menlo Park, CA), Ransom Hill Bioscience, Inc. (Ramona, CA), and Gibco BRL (Grand Island, NY). The human and rat β -actin primer pairs were obtained from Clontech Laboratories, Inc. (Palo Alto, CA), and amplify 838-bp and 764-bp PCR products, respectively.

Human A primer: 5'-ATG CTC ATG GCG TCC ACC

AC-3', sense (base pairs 72–91)

Human B primer: 5'-CTG AGA AGG AGC GAG AAG

GC-3', antisense (base pairs 564–583)

Human C primer: 5'-ACC GCT TCC GTG GGC

CAG AT-3', sense (base pairs 379–398)

Human D primer: 5'-GGA AGG CAG CTG AGC TTC

TC-3', antisense (base pairs 1221–1240)

Rat A primer: 5'-ATG GTG GGC ATG TAT GCC

TCC TCC TAC ATG-3', sense

(base pairs 399–428)

Rat B primer: 5'-AGT GTC ATC CTC ACG GTC TTG

GCC A-3', antisense (base pairs 835–859)

These primers were based on the published sequence for the human vasopressin V₂ receptor (7), the rat V₂ receptor (23), and human from previous studies (19,37). The human AB and CD primer pairs were chosen to span the two introns of the V₂ receptor gene so that DNA contamination of the samples could be ruled out. The location of the human primer pairs is shown in Fig. 1. The PCR mixture consisted of 2–10 μ l (~2–15 μ g) of template, 5 μ l of 10 \times PCR buffer II, 200 μ M of each deoxyribonucleotide triphosphate (dNTP), 0.5 μ M of each primer, and 2 μ M MgCl₂. The conditions used for PCR involved an initial step at 97°C for 8 min, and 30 cycles that included 30 s at 95°C, 1 min 30 s at 58°C, 1 min 30 s at 72°C, and a final step of 10 min at 72°C. PCR products were electrophoresed for 1 h at 100 volts using a 2% agarose gel and TAE buffer (Tris-acetate/EDTA electrophoresis buffer). In some PCR reactions 10 μ Ci/ml [α -³²P]dCTP (3000 Ci/mmol, Dupont/NEN, Boston, MA) was included, and the labeled products were electrophoresed at 100 volts for 4 h on a 4% polyacrylamide gel in TBE buffer (Tris-borate/EDTA electrophoresis buffer). Controls for RT-PCR included the omission of template, the cDNA for the vasopressin V₂ receptor as the template, and a 308- or 500-bp PCR positive control provided with the Gene Amp[®] kit (Perkin Elmer, Foster City, CA) or the SuperScript[®] preamplification system (GibcoBRL, Grand Island, NY).

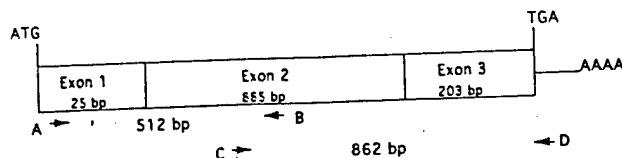


FIG. 1. Structure of the cDNA for the human vasopressin V₂ receptor and location of primers used for PCR amplification.

PCR products were cloned using the TA Cloning System and One Shot[®] competent cells (Invitrogen, San Diego, CA) according to the methodology provided by the manufacturer. DNA was purified using the Wizard[®] miniprep purification system (Promega, Madison, WI).

Northern Blot Analysis

Poly(A)⁺ RNA (5 μ g) from fetal human lung, adult human lung, and adult human kidney was electrophoresed on a 1.2% agarose formaldehyde denaturing gel for 3.5 h at 75 volts using 1 \times MOPS [3-(*N*-morpholino)propanesulfonic acid], and transferred overnight to a supported nitrocellulose membrane (MSI, Westborough, MA) using 20 \times SSC (saline sodium citrate, Boehringer Mannheim, Indianapolis, IN). The membrane was baked in a vacuum oven at 80°C for 2 h, and prehybridized for 3 h at 42°C in hybridization solution consisting of 5 \times SSC, 5 \times Denhardt's solution [0.1% (w/v) ficoll type 400, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin], 200 μ g/ml denatured salmon sperm DNA, 0.1% SDS, 6.25 mM NaH₂PO₄ (pH 6.5), and 50% formamide. The 862-bp CD PCR product amplified from the vasopressin V₂ receptor cDNA, and a 1.5-kb β -actin probe (ATCC, Rockville, MD) were labeled with [³²P]dCTP (3000 Ci/mmol) using exonuclease-free Klenow fragment and the DECAprime II[®] DNA labeling kit (Ambion, Austin, TX) to specific activities of approximately 1.7 \times 10⁸ cpm/ μ g and 1.9 \times 10⁸ cpm/ μ g, respectively. Hybridization was performed overnight at 42°C with 10⁶ cpm/ml of the [³²P]-labeled probe. Membranes were washed (1 \times 30 s with 2 \times SSC/0.1% SDS at 22°C; 2 \times 15 min with 2 \times SSC/0.1% SDS at 22°C; 2 \times 15 min with 0.1 \times SSC/0.1% SDS at 37°C; 1 or 2 \times 60 min with 0.1 \times SSC/0.1% SDS at 50°C) and exposed to X-ray film with intensifying screens for varying times (3–48 h).

DNA Sequence Analysis

DNA sequencing of the cloned AB and CD PCR products from adult human lung was performed using an Applied Biosystems Model 373A automated DNA sequencer, and AmpliTaq[®] DNA polymerase and the Taq DyeDeoxy[®] terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The primers used for sequencing were M13 forward, M13 reverse, and T7 universal primers, and the primers specific for the vasopressin V₂ receptor already described. Sequencing was performed for both strands, from at least three positive clones. Sequence analysis was performed using the BLAST network service (2).

RESULTS

Figure 1 indicates the location of the specific primers and expected sizes of the human AB and CD PCR products. As shown in Fig. 2, using the AB primers a 512-bp product was amplified from the cDNA for the vasopressin V₂ receptor (lane 1) and from adult human lung (lane 2). With the CD primer pair, a 862-bp product was amplified from the cDNA (lane 4) and

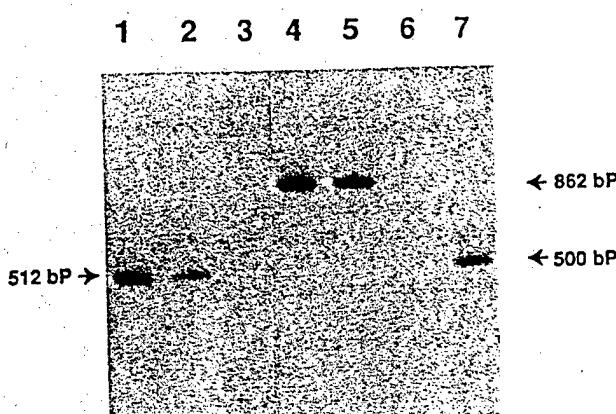


FIG. 2. RT-PCR analysis using reverse transcribed poly(A)⁺ RNA from adult human lung and primers specific for the vasopressin V₂ receptor. The experimental protocol is described in the Method section (10 μ Ci/ml [α -³²P]dCTP was included in the PCR reaction). Lane 1: cDNA for the vasopressin V₂ receptor as the template and AB primers. Lane 2: adult human lung and AB primers. Lane 3: no template and AB primers. Lane 4: cDNA for the vasopressin V₂ receptor as the template and CD primers. Lane 5: adult human lung and CD primers. Lane 6: no template and CD primers. Lane 7: PCR positive control. These results are representative of at least triplicate independent experiments.

from adult human lung (lane 5). As indicated in lanes 3 and 6, no products were amplified using the AB and CD primers in the absence of template. Sequencing of the AB and CD PCR products generated from the adult human lung confirmed sequence identity with the human vasopressin V₂ receptor (data not shown). Figure 3 shows a representative Northern blot with poly(A)⁺ RNA from fetal human lung, adult human lung, and adult human kidney using the 862-bp [³²P]-labeled CD PCR product as a probe. In the adult human lung (lane 2) and kidney (lane 3) a 1.9-kb mRNA species is apparent. However, in the fetal lung no significant band was detected using this probe. The membrane was rehybridized with a [³²P]-labeled β -actin probe to account for possible loading differences and sample integrity. Even though it appears that the fetal lung sample did not have a β -actin signal as strong as that of the adult lung and kidney, an obvious band for this mRNA is still apparent. In the duplicate Northern blot (data not shown), the same results were found with the exception that the β -actin signals were of similar intensity for the fetal lung and adult lung. As shown in Fig. 4, using RT-PCR and the CD primers the 862-bp PCR product was amplified from fetal human lung (lane 1), adult human lung (lane 2), and adult human kidney (lane 3). However, relative expression appears to be lower in fetal human lung than in adult human lung or kidney because the relative fluorescence from the 838-bp β -actin PCR product for all three samples appears to be of similar intensity (lanes 4–6). The other amplified PCR product from adult human lung (Fig. 4, lane 2), which appears to be 100 bp larger than the predicted size of the CD product, may reflect amplification from precursor hnRNA because the second intron of the V₂ vasopressin receptor gene is 106 bp. Also, both the Northern blot and RT-PCR data suggest that the relative expression of the mRNA for the vasopressin V₂ receptor in adult human lung is less than in the adult human kidney. As indicated in Fig. 5, using RT-PCR and primers specific for the rat vasopressin V₂ receptor a 461-bp PCR product was amplified from adult rat lung and kidney (lanes 1 and 2, respectively), and the relative fluorescence intensities of the 764-bp β -actin PCR products for rat

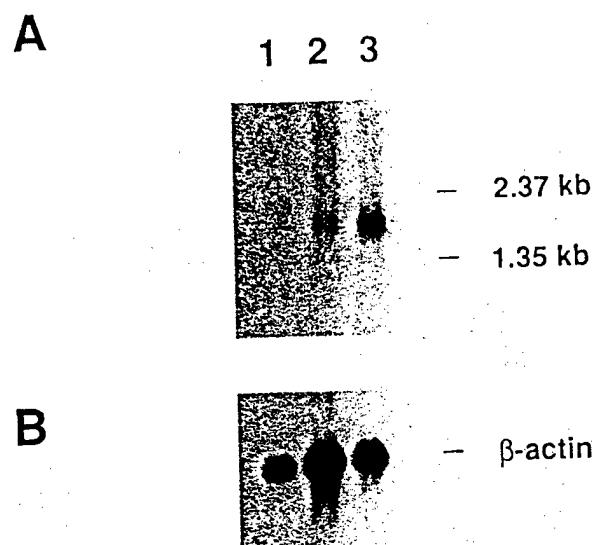


FIG. 3. Northern blot analysis for the vasopressin V₂ receptor using the 862-bp [³²P]-labeled CD PCR product as the probe and poly(A)⁺ RNA from human fetal lung, adult human lung, and adult human kidney. (A) Lane 1: poly(A)⁺ RNA from human fetal lung; lane 2: poly(A)⁺ RNA from adult human lung; lane 3: poly(A)⁺ RNA from adult human kidney. (B) The membrane was reprobed for β -actin. These results are representative of duplicate independent experiments.

lung and kidney (lanes 3 and 4, respectively) suggest that this mRNA is expressed more abundantly in rat kidney.

DISCUSSION

This is the first report, of which we are aware, demonstrating expression of vasopressin V₂ receptor mRNA in adult human

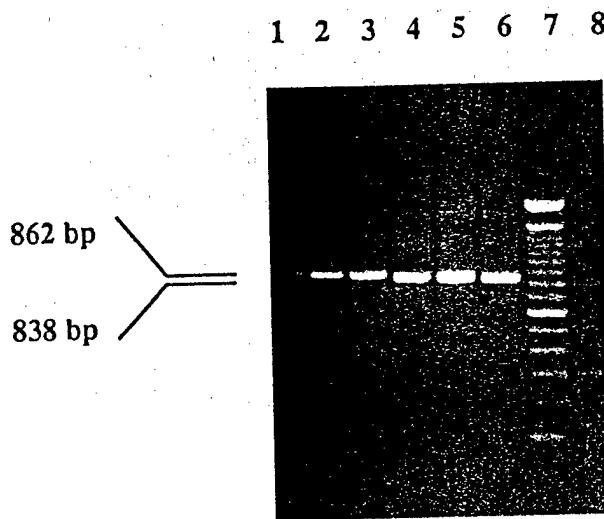


FIG. 4. RT-PCR analysis using reverse transcribed poly(A)⁺ RNA from fetal human lung, adult human lung, adult human kidney, and primers specific for the human vasopressin V₂ receptor and human β -actin. The experimental protocol is described in the Method section. Lane 1: fetal human lung and CD primers. Lane 2: adult human lung and CD primers. Lane 3: adult human kidney and CD primers. Lane 4: fetal human lung and β -actin primers. Lane 5: adult human lung and β -actin primers. Lane 6: adult human kidney and β -actin primers. Lane 7: 100-bp DNA ladder. Lane 8: PCR positive control. The gel was stained with ethidium bromide.

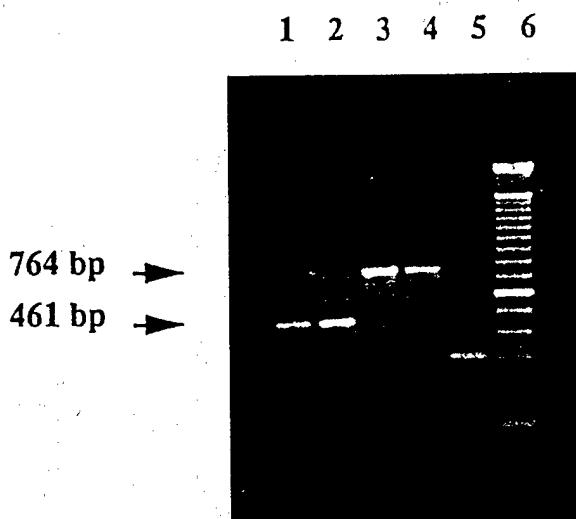


FIG. 5. RT-PCR analysis using reverse transcribed poly(A)⁺ RNA from adult rat lung and kidney, and primers specific for the rat vasopressin V₂ receptor and rat β -actin. The experimental protocol is described in the Method section. Lane 1: adult rat lung and AB primers. Lane 2: adult rat kidney and AB primers. Lane 3: adult rat lung and β -actin primers. Lane 4: adult rat kidney and β -actin primers. Lane 5: PCR positive control. Lane 6: 100-bp DNA ladder. The gel was stained with ethidium bromide.

lung. Other researchers have described the extra-renal expression of the vasopressin V₂ receptor in rat brain (19,20), and radioligand binding studies indicate that human mononuclear phagocytes and platelets have vasopressin V₂ receptor-like binding sites (6,8). Our data indicate that adult human lung expresses the mRNA for the vasopressin V₂ receptor. Northern blot indicates that this mRNA is of a similar size to that of the human kidney, and the 1.9-kb size is similar to that found by other researchers for the V₂ receptor mRNA (7). DNA sequencing of the cloned PCR products revealed that the V₂ receptor mRNA from adult human lung has exact identity with the published sequence for the cloned vasopressin V₂ receptor (7).

In contrast with the adult human lung, Northern blot evaluation indicates that the fetal human lung did not express detectable levels of mRNA for the vasopressin V₂ receptor. However, using the more sensitive RT-PCR technique, a PCR product of the predicted size was amplified from fetal human lung. Relative expression of this mRNA in fetal human lung appeared to be lower than in adult human lung or adult human kidney. The fetal lung mRNA used in this study was obtained from 22–24-week fetuses. Because the duration of human gestation is between 37 and 41 weeks, one potential explanation for the low level of expression may be that the receptor mRNA is not actively transcribed until a time closer to birth. Because the critical role for the vasopressin V₂ receptor in fetal lung is thought to involve removal of fluid from the lung in preparation for the transition

from a uterine to an air-breathing environment (18,28–30,32,38), this explanation seems plausible. Research using fetal sheep adds support to the idea of the developmentally regulated expression of lung vasopressin receptors, because vasopressin was unable to inhibit lung liquid secretion until 135 days of gestation in these animals (38).

Using total RNA and RT-PCR with primers specific for the rat V₂ receptor, other researchers were unable to amplify a PCR product from lung tissue of fetal and adult Sprague–Dawley rats (19). Using the same primers and Poly(A)⁺ RNA from adult Sprague–Dawley rat lung and kidney, PCR products of the predicted size of 461 bp were obtained. This discrepancy between our findings for adult rat lung and the previous findings may reflect differences in PCR conditions, or that the V₂ receptor mRNA in rat lung is expressed in low levels and only detectable by RT-PCR with Poly(A)⁺ RNA selection. Our data therefore suggest that vasopressin V₂ receptors may also play a role in rat lung physiology, and that rats may be a suitable model for examining the developmental expression of vasopressin V₂ receptors in the lung. Other researchers have found no effect of arginine vasopressin on cAMP production and ion transport in fetal rat distal lung epithelium (27). These data may reflect an insufficient expression of the vasopressin V₂ receptor in fetal rat lungs at this stage of development (day 20, term is day 22) in Wistar rats, the strain used by these investigators. An alternative explanation is the loss of functional V₂ receptors during the isolation of primary cultures.

Numerous physiological studies have suggested the presence of vasopressin V₂ receptors in fetal sheep, goat, and guinea pig lung tissue, because epinephrine and other agents that activate cAMP have an effect similar to vasopressin on decreasing lung liquid secretion (4,9,22,28,29,39,40). The actions of vasopressin in the lung at these V₂ receptors is attributed, at least in part, to activation of apical amiloride-sensitive sodium channels (13,30,34). However, the action of vasopressin on an aquaporin-like water channel cannot be discounted, because aquaporin-5 has recently been isolated from distal human lung (31). Like aquaporin-2, the water channel regulated by vasopressin in the kidney (14,15,24), aquaporin-5, has a cAMP–protein kinase consensus sequence. In addition, aquaporin-5 shares 63% amino acid homology with aquaporin-2. The physiological role for the vasopressin V₂ receptor in adult human lung is not known. However, it is reasonable to assume that, as in the kidney, this receptor plays a role in fluid balance.

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Ionic Signals in T47D Human Breast Cancer Cells

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ABSTRACT. Increasing evidence that ion channels play a key role in the modulation of cellular mitogenesis led us to investigate the membranes of T47D human breast cancer cells to identify the ion currents present. We report here the results of voltage-clamp studies in the whole-cell configuration on isolated, non-synchronized single cells obtained from a ductal breast carcinoma. In these studies we identified an outward rectifying potassium current and a chloride current. The potassium current activated at potentials more positive than -40 mV, reached an average value of 1.4 nA, and did not inactivate with time. This current was sensitive to block by extracellular tetraethylammonium chloride (TEA, $IC_{50} = 1$ μ M), was insensitive to charybdotoxin (CTX, $IC_{50} = 7.8$ μ M), and was not diminished by repetitive pulses separated by 1 s. Rapid voltage-dependent inactivation of the current was demonstrated by tail current analysis. The current appeared calcium-insensitive. Application of hyperpolarizing pulses did not elicit an inward potassium rectifier current. Treatment with tetrodotoxin did not reveal the presence of an inward sodium current. The potassium current was increased by the presence of aspartate in place of chloride and in the presence of the chloride channel blocker 4,4'-dilothiocyanostilbene-2,2'-disulfonic acid (DIDS). We conclude that currents present in T47D breast cancer cells include a chloride current and a voltage-gated potassium outward rectifier. We suggest that the potassium current, either alone or in conjunction with potassium currents reported in different human breast cancer cell lines by others, may play a role in the modulation of the cell cycle. *CELL SIGNAL* 8:279-284, 1996.

KEY WORDS. Ion channels, Breast cancer, Potassium currents, Mitogenic signals

INTRODUCTION

An increasing body of evidence supports the hypothesis that potassium currents signal cellular proliferation [1]. Small-cell lung carcinoma cells (SCCL) treated with the K^+ -channel antagonist 4-aminopyridine (4-AP) demonstrate an attenuated outward K^+ current coincident with a decrease in cell proliferation [2]. In MCF-7 breast cancer cells the potassium channel antagonists quinidine, glibenclamide, and linoglizide inhibit cell proliferation and cause the accumulation of cells in the G_1/G_0 phase of the cell cycle [3]. This effect on MCF-7 cells was attributed to the putative activity of adenosine triphosphate (ATP)-sensitive potassium channels. This cell line, established from pleural fluid of a patient with adenocarcinoma, retains certain characteristics of differentiated epithelium, including the presence of estrogen receptors [4]. A calcium-activated potassium current has been identified in MCF-7 cells [5] that, although correlated with cell proliferation, was not considered obligatory for growth [6].

The T47D breast cancer cell line selected for this study, originally established from the pleural effusion of a patient with infiltrating ductal carcinoma [7], also displays characteristics of an epithelial origin. These cells possess receptors for estrogen [8, 9], progesterone [6, 10-12], calcitonin [13], and vitamin D [14, 15]. We selected this cell line for investigation because estrogen-resistant clones have been produced, and we aim to develop this line as a model for evaluating breast cancer progression [16-18]. In view of the previous studies that have linked ion channels with mitogenesis, we initiated this study to identify the whole-cell currents present in unsynchronized cells of the T47D human breast cancer cell line.

MATERIALS AND METHODS

Cell Culture

The T47D human breast ductal carcinoma cell line was obtained from the American Type Culture Collection (ATCC HTB133, Rockville, MD), and maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 0.2 IU bovine insulin/ml (Sigma, St. Louis, MO) and 10% FBS.

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fetal bovine serum (FBS; Hyclone Labs, Logan, UT). Cells received fresh growth medium or were subcultured (1:3 using 0.25% trypsin + 0.02% ethylenediamine tetraacetic acid (EDTA)) every 2-3 d.

Cell Preparation

Cells were plated onto glass coverslips approximately 18 h before patch-clamp studies were initiated. Each coverslip was placed in a chamber of 0.2 mL volume and washed with extracellular physiological solution. The chamber was placed on the stage of an inverted Nikon microscope and viewed with Hoffman optics ($\times 640$). Electrode preparation and other experimental details have been presented previously [19-21].

Solutions

The physiological external bath solution for recording whole-cell currents contained the following (in mM): 140 NaCl, 4.5 KCl, 2.0 CaCl₂, 1.0 MgCl₂, and 10 4-(2-hydroxyethyl)-1-piperazine-N'-2-ethanesulfonic acid (HEPES), pH balanced to 7.3 with NaOH. The pipette solution contained (in mM) 140 KCl, 1.1 ethylene glycol-bis-(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 CaCl₂, 2.0 MgCl₂, and 10 HEPES, pH balanced to 7.3 with KOH. [Ionized Ca²⁺] = 10⁻⁶ M. All experiments were done at room temperature (22°C). Extracellular KCl was increased by substituting KCl for NaCl in the bath solution. Extracellular Cl⁻ was reduced by substituting potassium aspartate for KCl in the bath. Intracellular [Ca²⁺] was increased by changing pipette solution CaCl₂ to 1.08 mM [free Ca²⁺] = 4 \times 10⁻⁶ M.

Blocking Agents

Tetraethylammonium chloride (TEA; Sigma) was dissolved in extracellular solution and added in different concentrations directly to the bath. Charybdotoxin (CTX) from *Leiurus* venom was obtained from Alomone Labs (Jerusalem, Israel). The CTX was dissolved in 0.1% BSA, 100 mM NaCl, 10 mM Tris (pH 7.5) and 1 mM EDTA. This solution required the addition of 0.01% bovine serum albumin (BSA) to the bath. Because the bath total volume was 0.2 mL, replacement of the bath required only two drops of fluid. Replacement of the bath effected rapid and complete mixing of experimental solutions, as demonstrated by addition of a water-soluble dye, 4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), a recognized Cl⁻-channel blocking agent, was added to the bath.

Data Acquisition and Analysis

Currents in the individual cells were measured by rupturing the cell membrane in the lumen of the patch electrode by suction. Patch-clamp data were obtained with a List-EPC 7 clamp circuit (Medical Systems, Greenvale, NY). Experimental protocols, details of which are presented in the figure legends, were written for PCLAMP software (Axon Instru-

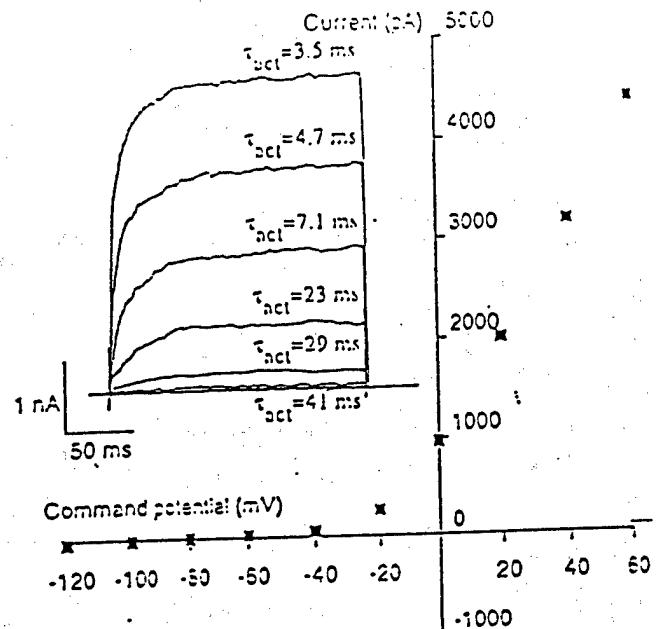


FIGURE 1. Outward rectifying K⁺ current in T47D breast cancer cells. Current vs. voltage curve showing peak outward currents recorded in a cell held at -80 mV and then stepped to voltages from -120 mV to +60 mV. Inset: Currents recorded from this cell. Note the lack of time-dependent inactivation. The time constant for activation (τ_{act}) was best described by a single exponential for voltage steps to -40, -20, and 0 mV, and by a double exponential at more positive depolarizations. The fast time constant is shown for each trace.

ments, Burlingame, CA). Data were digitized at 5 KHz and recorded with a 486 PC computer after filtering with an 8 pole Bessel filter at 1 KHz. Series resistance correction, capacity compensation, and leakage subtraction were done. All data are reported as mean \pm S.E.M.

RESULTS

Patch-clamp recordings and analyses of T47D human breast cancer cells revealed the presence of a voltage-sensitive current. This current was identified as a voltage-gated, time-independent, TEA-sensitive, charybdotoxin-insensitive, outwardly rectifying potassium current, and was present in 71 of 79 cells studied. We found no evidence of an inward rectifying potassium current or of a sodium current.

Voltage-gated Potassium Current

After rupture of the cell membrane by suction, voltage steps were applied to the cell in a normal physiological ionic gradient from a holding potential of -80 mV to command potentials of -120 mV to 60 mV. The large outward currents elicited are shown in the inset of Figure 1. The current was activated at -39.7 ± 2.2 mV ($n = 10$) and showed no time-dependent inactivation. At +40 mV depolarizations, the current reached a peak value of $1,376 \pm 231$ pA ($n = 20$). The time constants for activation could be represented by a single exponential at more negative voltage levels and

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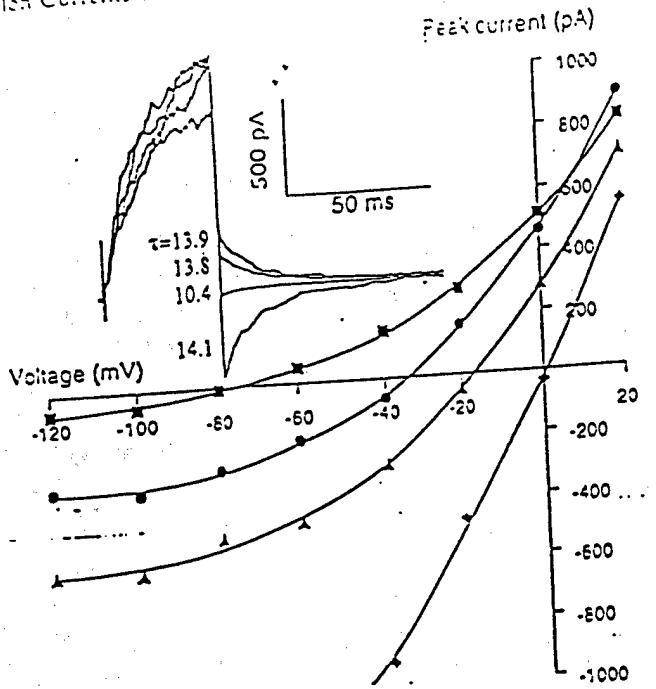


FIGURE 2. Tail currents. Peak current vs. voltage curves for tail currents recorded after a step depolarization from -80 mV to $+40$ mV. Pipette KCl concentration = 140 mM. External KCl concentration = 4.5 mM (squares), 38 mM (circles), 72 mM (triangles), or 140 mM (diamonds). Inset: Tail currents recorded at -20 mV in each external KCl concentration, with single exponential time constants (τ).

by a double exponential at more positive depolarizations. The fast time constants for activation are indicated for each current trace.

Tail Current Analyses

The outward current was activated by a voltage step from a holding potential of -80 mV to $+40$ mV. V_m was then stepped to voltages between $+20$ and -120 mV and tail currents were recorded as shown in Figure 2. Rapid voltage-dependent inactivation was observed. Changing bath KCl concentration produced changes in reversal potential for the outward current. In external KCl concentrations of 4.5 , 38 , 72 , and 140 mM, E_{rev} was -84.5 ± 1.2 mV, -31.8 ± 1.7 mV, -17.6 ± 0.2 mV, and -2.6 ± 2.2 mV, respectively ($n = 7$). For these KCl concentrations, calculated E_{rev} for a K^+ -selective current was -86.6 mV, -32.8 mV, -16.7 mV, and 0 mV, respectively. Thus, this current appears to be carried by K^+ ions. The rapid voltage-dependent recovery of the current is emphasized by the absence of use-dependent peak amplitude reduction when cells were repetitively depolarized at intervals of 1 s ($n = 8$).

Voltage Ramps

After holding the membrane potential at -80 mV, voltage ramps were applied to the cell membranes from -120 mV to $+40$ mV, with the resulting I vs. V graphs shown in

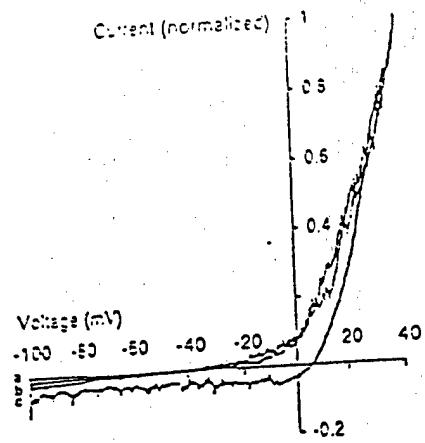


FIGURE 3. Voltage ramps in T47D breast cancer cells. From a holding potential of -80 mV, voltage ramps from -100 to $+40$ mV are shown. Curve a shows standard solutions. In curve b, pipette Ca^{2+} concentration was increased. The similarity between curves a and b suggests that no calcium-activated currents were present. Increasing external KCl concentration to 140 mM (c) alters the current vs. voltage relationships as expected. No evidence of an inwardly rectifying K^+ current is seen, although the increase in leakage current suggests the presence of other non-voltage-activated currents.

Figure 3. These experiments were performed with standard pipette and bath solutions, and also after increasing pipette Ca^{2+} concentrations to levels as high as 4×10^{-6} and external KCl concentrations to 140 mM. In no case was a calcium-activated or inwardly rectifying current detected. However, the increased leakage current noted in symmetrical KCl solutions suggests the presence of additional K^+ currents, perhaps a ligand-modulated current or the ATP-sensitive current reported by Woodfork et al. [3].

TEA

Figure 4 illustrates the results of 5 experiments in which voltage steps from -80 mV (holding) to $+20$ mV were applied in the presence of increasing concentrations of TEA chloride. Exponential curve fitting allowed calculation of a 50% inhibitory concentration (IC_{50}) of $1 \mu M$ TEA chloride, indicating a marked sensitivity of the current to this substance.

CTX

Charybdotoxin, a toxic component isolated from the venom of the scorpion *Leiurus*, has been shown in a number of studies to selectively block calcium-activated K^+ channels and to have a strong blocking action on other types of K^+ channels. Results of 5 experiments in which cells were depolarized after exposure to CTX are shown in Figure 5. Calculated IC_{50} was $7.8 \mu M$, demonstrating an insensitivity of the current to the blocking effects of CTX. Both the solvent and BSA were tested in the absence of CTX and were found to have no effect on currents. The solution containing CTX completely replaced the bath in order to assure complete mixing.

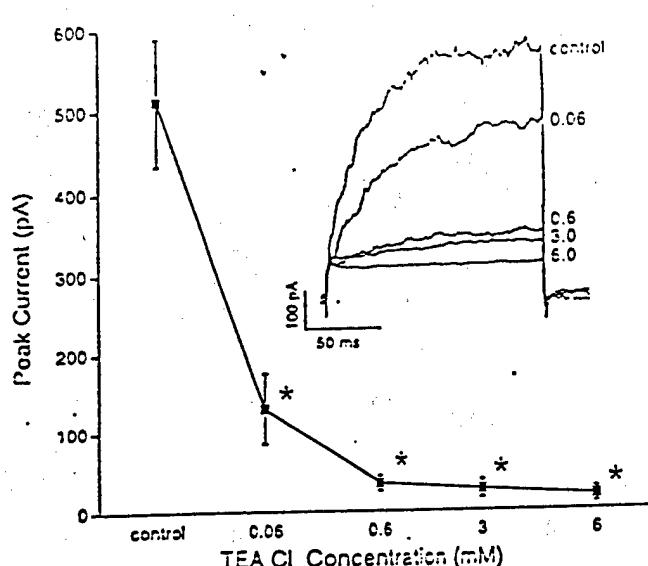


FIGURE 4. Effects of TEA chloride. Dose-related reduction in peak current following exposure to TEA chloride is demonstrated. Inset: Typical current recordings of a step depolarization from -80 mV to $+20$ mV after exposure to increasing TEA Cl concentrations. $*P < 0.0001$ vs. control by one-way ANOVA for repeated measures and Tukey's Honestly Significant Different Test.

Inward Currents

SODIUM. To determine whether a sodium inward current was present, we added TTX (3×10^{-6} M) to the bath solution. The membrane was subjected to a more negative holding potential to remove any inactivation that might be present, and the membrane was then depolarized by step pulses. No effect on the whole-cell current was detected, leading us to conclude that a voltage-gated sodium current is not present.

POTASSIUM. Experiments were designed to test for the presence of an inward potassium rectifier. The membrane

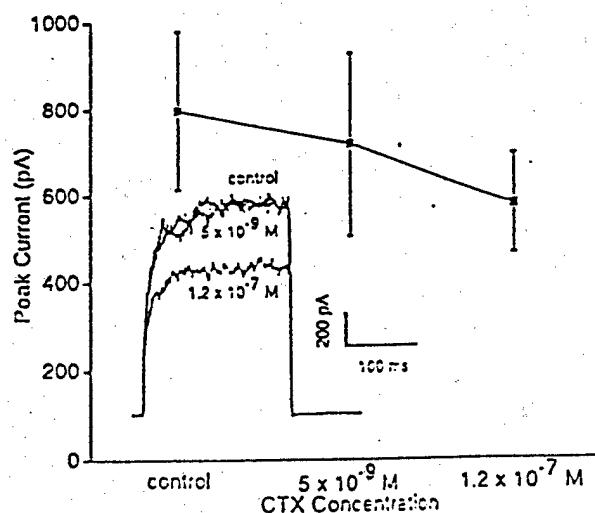


FIGURE 5. Effects of CTX. Increasing concentrations of CTX produce minimal decreases in peak outward current following a depolarization of -80 mV to $+20$ mV ($P = 0.11$). Inset: Typical current recordings after exposure to each concentration of CTX.

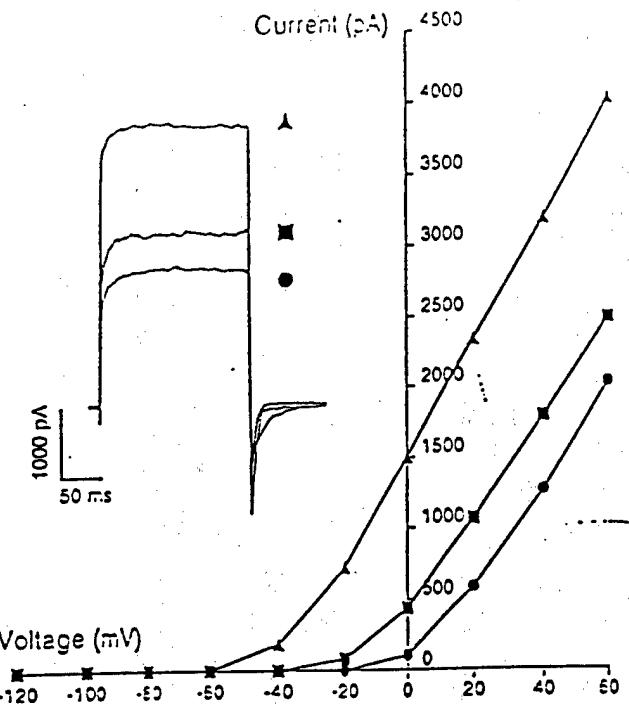


FIGURE 6. Augmentation of outward current by aspartate. From a holding potential of -80 mV, voltage steps from -120 to 60 mV were applied. The inset displays currents recorded after voltage steps to 60 mV in physiological bath solution (square), symmetrical 140 mM KCl solution (circle), and after substitution of 140 mM K aspartate for KCl (triangle). Current-voltage relationships for the same experiments demonstrate a marked increase in outward current after aspartate substitution.

was stepped to various hyperpolarized potentials in physiological, reversed, and altered extracellular potassium concentrations. There were no currents detected under these conditions.

CALCIUM. Increased amounts of calcium delivered both to the pipette and to the bath did not effect any changes in the outward current. With potassium replaced by CsCl in the pipette and with TEA in the bath, increased calcium in and out and addition of BaCl_2 (20 mM) did not reveal any inward current. Addition of CTX did not change the magnitude or time course of the potassium current. We conclude that calcium does not effect this voltage-gated potassium current.

CHLORIDE. The substitution of aspartate for chloride in the bath solution greatly enhanced the potassium current, as shown in Figure 6. The membrane potential was held at -80 mV, after which depolarizing steps were delivered from -120 mV to 60 mV. The marked increase in the total outward current is shown in the current traces and in the I vs. V relationship.

Chloride substitution experiments, tested by tail-current analyses, gave further evidence of the chloride component of the total current, as shown in Figure 7A-D. The cell membrane was held at -80 mV and then depolarized to $+40$ mV. Tail currents were then measured as the membrane

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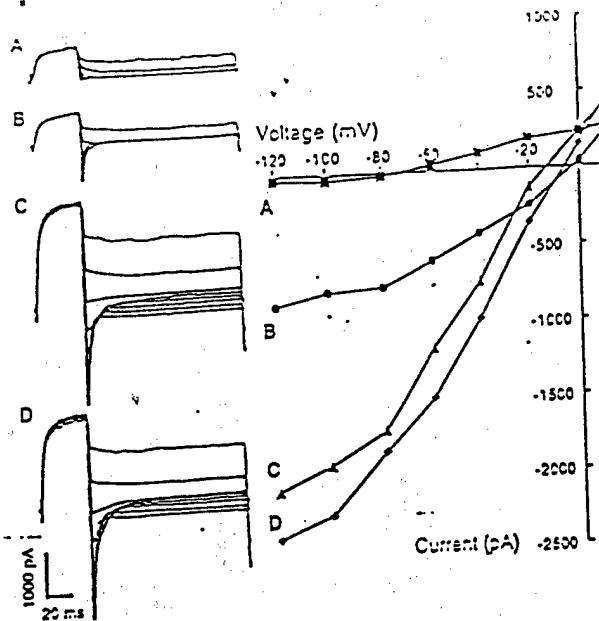


FIGURE 7. (A-D) Effects of chloride substitution on tail currents. Current traces A, B, C, and D display tail currents recorded after a depolarization to 40 mV from a holding potential of -80 mV. Shown are tail currents produced by voltage steps from 20 to -120 mV. The current-voltage curves for these traces are shown at the right. The pipette contained 140 mM KCl. Panel A was recorded in physiological bath solution. Panel B was recorded in symmetrical KCl solution and demonstrates a reversal potential near 0 mV. In C, 140 mM K aspartate was substituted for KCl in the bath solution and a change in reversal potential occurs. In D, 70 mM KCl with 70 mM K aspartate produced an intermediate change in reversal potential.

was stepped from 20 to -120 mV in a normal physiological gradient (Fig. 7A). The potassium content of the bathing solution was then elevated so that the potassium gradient was symmetrical (Fig. 7B). Under this condition, the reversal potential was near 0 mV, (I vs. V graph), as one would predict for a potassium current. The reversal potential was then observed to shift as K⁺ aspartate was substituted for KCl (Fig. 7C). The reversal potential again shifted along the voltage axis as the bath was changed to contain 70 mM KCl and 70 mM K aspartate (Fig. 7D).

An increase in the outward current was also measured when the chloride channel blocker DIDS was added to the bath. Application of a continuous ramp of increasing voltage (-140 to 40 mV) from a holding voltage of -80 mV resulted in an increase in the outward current from 1,042 pA to 1,265 pA, as shown in Figure 8. We conclude from these data that a chloride current is present in these cells.

DISCUSSION

The voltage-gated potassium current we have characterized in T47D breast cancer cells activates rapidly at a membrane voltage of -40 mV, is outwardly rectifying, and displays no time-dependent inactivation. Peak currents are very large and at a depolarization level of 40 mV (holding: -80 mV), reached levels of 1.4 nA. This current is sensitive to block

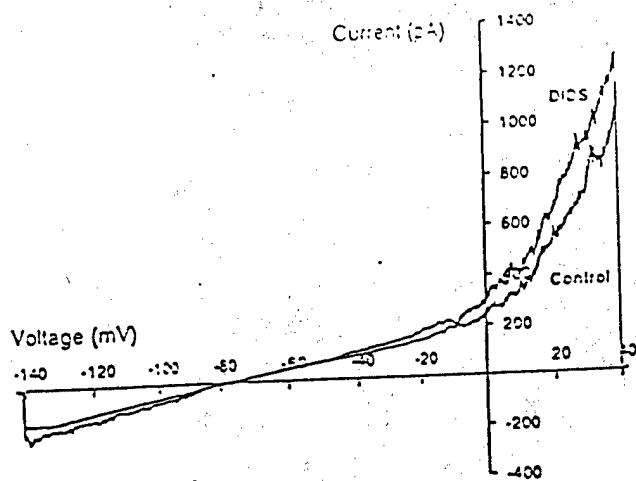


FIGURE 8. Increase in outward current after addition of DIDS. From a holding potential of -80 mV, a continuous ramp of increasing voltage from -140 mV to 40 mV was applied. The pipette contained 140 mM KCl. During the control recording, the bath contained standard physiological solution. After the addition of DIDS 4×10^{-4} M to the bath, peak current increased from 1,042 pA to 1,265 pA. This suggests that DIDS blocked an inward Cl⁻ current.

by TEA but not to CTX. TEA is a widely documented blocker of a potassium channels, but CTX is now known to block other potassium channels as well as the calcium-activated potassium channels [22]. In some cells, CTX blocks even the voltage-gated maxi-K channel [23]. In the experiments reported here, increased levels of internal calcium did not effect any change in either the magnitude of the potassium current or in the reversal potential, results that indicate that a calcium-activated potassium current is either not present or is of such relative magnitude compared to the outward rectifier that it is not readily visible by the whole-cell technique. In experiments in which the membrane holding potential was increased to hyperpolarized levels of -140 mV, no evidence of an inward rectifier was seen. The reversal potentials measured in a range of transmembrane potassium concentrations compared with the calculated values provide clear evidence for the ionic identity of the charge carrier. This current was not changed in the presence of tetrodotoxin, an observation that supports our conclusion that a sodium current is not present.

This report documents the first recordings of electrical activity from the T47D cell line of human breast cancer, an epithelial-like cell line obtained from an infiltrating ductal carcinoma of the breast. Another epithelial-like cell line of human breast cancer (MCF-7), obtained from an adenocarcinoma, has been explored by Woodfork et al. [3]. They concluded, from experiments using known potassium blockers on cell proliferation, that a ligand-activated potassium channel (i.e., an ATP-sensitive channel) is most likely involved in the proliferation of MCF-7 cells. In a preliminary report of voltage-clamp experiments [24], three types of current/voltage relations were described in MCF-7 cancer cells. In the presence of Mg-UDP (uridine diphosphate to enhance activation of K_{ATP}) in the pipette, linear I vs. V relationships

showed reversal potentials of -62 mV and -7 mV, respectively, while a third current was outwardly rectifying and reversed at -23 mV. Only the third type was observed when 2 mM ATP was also added to the pipette. The only current reported that appears similar to the outward rectifier we describe in T47D cells is a linear current with a reversal potential of -62 mV. The major potassium current in these two cell lines seems to be remarkably different. While we have not yet specifically studied the effects of ATP, we did note an increased leakage current in symmetrical potassium, which suggests the presence of an additional component.

Evidence is presented here for the presence in T47D cells of a chloride channel. The data include enhancement of the outward current by aspartate substitution for chloride, enhancement of the outward current in the presence of the chloride blocking agent DIDS, and the shift of the reversal potential in the presence of altered chloride concentrations in the bath. These data are consistent with the criteria for the identification of a chloride current.

In view of the complex nature and behavior of breast cancer cells, it is not difficult to appreciate that chloride ions may function in multiple roles (e.g., membrane stabilization, regulation of intracellular pH, modulation of secretion, and cell volume control). These cells exhibit prolific secretory behavior. Another possibility to be considered in regard to this chloride current is that the aspartate itself may unmask a different potassium component, since a variety of amino acids are known to exert excitatory effects on cells that have receptors for these agents [25]. Further studies should help to characterize and identify this component of the whole-cell currents.

Whether the apparent differences in the currents measured in the MCF7 and T47D cell lines are related to some basic property of these two types of cells or to variations in the stage of the cell cycle may be resolved in future studies using cell-cycle-synchronized cultures. Based on previous studies with other cell types [1], we suggest that it is more likely that the potassium current will be involved in modulation of the cell cycle while the chloride channel will be linked to secretion, regulation of cell volume, or some other cellular function.

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Immunohistochemical evaluation of vasopressin gene expression in fibrocystic breast disease.

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Summary: We previously found that expression of the vasopressin gene is a common feature of human breast cancer. In the present study we examined 17 different cases of benign fibrocystic breast disease for vasopressin expression using immunohistochemistry and antibodies directed against vasopressin and vasopressin-associated glycopeptide. All cases examined were negative for vasopressin gene expression using these antibodies. These results suggest that vasopressin gene expression occurs as part of the carcinogenic process rather than being a marker of cellular proliferation in the breast.

Key Words: Vasopressin, fibrocystic breast disease, immunohistochemistry

Running Title: Vasopressin and fibrocystic breast disease

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Introduction

Although a number of risk factors have been identified as possible causative agents for breast cancer, the etiological origin of this disease remains obscure (Henderson, 1993). Among those conditions which are predisposing towards breast cancer are proliferative breast disease, particularly atypical ductal and lobular hyperplasia (Dupont and Page, 1985; London et al., 1992; Connolly and Schnitt, 1993; Dupont et al., 1993). Atypical hyperplasia is classified as a borderline lesion because it has some of the histological features of carcinoma *in situ*. Previously we found that expression of the vasopressin gene is a common feature of human breast cancer using immunocytochemistry and antibodies directed against different regions of the vasopressin precursor (North et al., 1995). These results lead to the possibility that vasopressin expression could either, be a marker of cellular proliferation, represent part of the oncogenic process, or be a recognizable feature of cancer progenitor cells in precancerous breast lesions. We have commenced efforts to examine these questions by performing an immunohistochemical evaluation for vasopressin gene-expression using archival material representing various fibrocystic breast lesions.

Materials and Methods

Tissues

Formalin-fixed biopsy specimens were obtained from 17 patients with various forms of benign breast disease who were examined between 1975 - 1984 at Dartmouth Hitchcock Medical Center (DHMC, Lebanon, NH). The cases included, 4

cases of fibrocystic disease without hyperplasia, 9 cases of fibrocystic disease with ductal or lobular hyperplasia, and 4 cases of fibrocystic disease with atypical ductal hyperplasia. Diagnosis from pathology reports was confirmed by examining hematoxylin- and eosin- stained sections. These cases were followed for the subsequent development of breast cancer. Formalin-fixed specimens of human hypothalamus and pituitary were obtained from autopsies performed at DHMC.

Antibodies

Rabbit polyclonal antibodies directed against vasopressin and the 18 amino acid C-terminal vasopressin-associated glycopeptide were prepared using previously published methods (North et al., 1991; Friedmann et al., 1994). Antibody purification involved ammonium sulfate precipitation of the immunoglobulin fraction, and fractionation on a column of protein A Sepharose with pH gradient elution (pH 7.6 - pH 3.0). Antibodies were obtained as a pH 4.0 subfraction, dialyzed and lyophilized. Based on dilution trials, antibodies to vasopressin and vasopressin-associated glycopeptide were used at concentrations of 11 ng/ml (1:2,000) and 190 ng/ml (1:800), respectively. Protein concentrations were determined using differential spectroscopy (Waddell, 1956).

Immunohistochemistry

Sections of 4 - 6 microns from each specimen of fibrocystic breast disease were deparaffinised and stained for vasopressin and vasopressin-associated glycopeptide using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA,

USA) and avidin-biotin complex (ABC) immunohistochemistry (Guesdon et al., 1979). Tissues were rehydrated by washing with xylene, descending concentrations of ethanol, and PBS (2 x 3 min, ambient temperature). Slides were blocked with 10% normal goat serum in PBS for 20 min at ambient temperature. The blocking solution was aspirated and sections incubated overnight at 4 °C with primary antibody diluted in PBS with 1.5% goat serum. Following incubation with primary antibody the slides were washed with PBS (2 x 3 min). Goat anti-rabbit biotinylated secondary antibody diluted in PBS containing 1.5% goat serum was applied at a concentration of 20 µg/ml for 30 min. Unbound secondary antibody was removed by washing 2 x 3 min with PBS, and endogenous peroxidase activity blocked using 3% hydrogen peroxide dissolved in absolute methanol (Streefkerk, 1972). After washing with PBS (3 x 5 min), slides were incubated with the avidin-peroxidase complex (25 µg/ml) for 30 min at ambient temperature. Slides were washed with PBS (2 x 3 min), and visualization of bound complex was achieved by adding a solution of 3,3'diaminobenzidine (0.2 mg/ml in PBS with 0.03% hydrogen peroxide) for 2 - 5 min. Tissues were then counterstained with hematoxylin, dehydrated in ascending concentrations of ethanol, washed in xylene, and coverslipped using permount. Antibody specificity was insured by incubating negative controls with pre-immune rabbit serum fractionated, using protein A Sepharose, at pH 4.0.

Results

Positive immunohistochemical staining was obtained for vasopressin neurons in human hypothalamus and for neuronal terminals of these neurons in the posterior

pituitary with both antibody preparations (data not shown). Alternatively, negative staining was obtained with both of these antibodies in the 17 cases of benign breast disease. In several tissue sections staining of mononuclear cells was evident. Staining of sections with pre-immune rabbit serum resulted in a lack of staining. As demonstrated previously, Fig 1a demonstrates positive staining of an acetone-fixed infiltrating ductal breast cancer biopsy specimen with the vasopressin-associated glycopeptide antibody (North et al., 1995). In this section the breast cancer cells demonstrate intense staining, and the normal ducts of the breast are unstained. Figure 1b demonstrates a tissue section of fibrocystic disease with adenosis which showed no immunostaining with the antibody to vasopressin-associated glycopeptide. A case of atypical ductal hyperplasia is represented in Fig 1c which exhibited negative staining with the antibody to vasopressin. It should be noted that the nuclei of cells appear dark because the nuclear counterstain hematoxylin was used. Follow-up of the medical records revealed that three individuals, one from each classification group, subsequently developed breast cancer.

Discussion

There is an increasing body of evidence which supports a connection between vasopressin and breast cancer. There are two published clinical reports of patients with breast cancer presenting with the syndrome of inappropriate antidiuretic hormone secretion (Gupta et al., 1986; Howard et al., 1993). Both *in vitro* and *in vivo* studies support a connection between vasopressin and breast cancer. Several studies indicate that a cell line derived from a dimethylbenz(a)anthracene-induced rat

mammary tumor possesses functional V_{1a} vasopressin receptors (Monaco et al., 1978; Monaco et al., 1980; Guillon, et al., 1986; Kirk et al., 1986; Woods and Monaco, 1988). Vasopressin was shown to have a growth-promoting influence on MCF-7 breast cancer cells, presumably through V_1 vasopressin receptors (Taylor et al., 1990). Another study has demonstrated that vasopressin induces a rise in intracellular free calcium in a human breast cancer cell line (Bunn et al., 1992). In a transgenic mouse model of breast cancer, ectopic vasopressin was found to stimulate cancer growth, but did not influence the time to tumor onset (Chooi et al., 1994).

Using antibodies directed against various regions of the vasopressin prohormone and the technique of immunohistochemistry we found that expression of vasopressin gene-related products is a common feature of breast cancers (North et al., 1995). In the present study it was found that the various cases of fibrocystic disease did not exhibit staining with antibodies directed against vasopressin or vasopressin-associated glycopeptide. These two antibodies had previously given positive immunostaining with all of the breast cancer specimens examined. The results obtained in the present study suggest that expression of vasopressin gene-related products is associated with the carcinogenic process and not with benign breast disease. These results also indicate that expression of vasopressin gene-related products is not just a marker of proliferation in the breast as indicated by the lack of staining of the tissue sections consisting of hyperplasia. Examination of subsequent medical records for these patients indicated that 3 of these cases (1 case of fibrocystic disease without hyperplasia, 1 case of fibrocystic disease with

hyperplasia, 1 case of fibrocystic disease with atypical hyperplasia) went on to develop breast cancer. Although the sample number is small these findings suggest that vasopressin gene-related products are not markers of premalignant lesions which will subsequently develop into breast cancer. Such findings are consistent with those obtained by Chooi et al. (1994) with MMTV-VP vasopressin transgenic mice. In these animals it was observed that vasopressin had no influence on normal mammary gland function and development, and did not cause the development of hyperplastic alveolar nodules and ductal hyperplasia. Taken together, these data are suggestive that vasopressin gene expression is not involved in benign breast disease, and is not a marker of preneoplastic changes in the breast. It would therefore seem to be a marker of the carcinogenic process in the breast. We are in the process of examining cases of carcinoma *in situ* to determine if vasopressin gene-related products are detectable at this stage of breast cancer.

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Figure 1 a, Positive staining for vasopressin-associated glycopeptide in a tissue section of infiltrating ductal breast cancer. Note the lack of staining in a normal structure, as indicated by the arrow (magnification = 147 x). b, Negative staining for vasopressin in a tissue section of fibrocystic disease with atypical ductal hyperplasia (magnification = 147 x). c, Negative staining for vasopressin-associated glycopeptide in a tissue section of fibrocystic disease with adenosis (magnification = 147 x).

TISSUE SPECIFIC EXPRESSION OF HVACM/CUL-5, A CULLIN PROTEIN, IN HUMANS

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Summary

HVACM, also known as Cul-5, is a member of the cullin protein family. Cullins are involved in the ubiquitin-mediated degradation of cell cycle proteins, but the precise function(s) of Cul-5, a protein that interacts with the hormone vasopressin, is unknown. We developed polyclonal antibodies against this protein, and looked for

Cul-5 expression in AMeX-processed normal human tissues. Cul-5 is expressed intracellularly in kidney distal convoluted tubule, skeletal muscle capillary endothelial cells, Kupffer cells of the liver, and by cells inhabiting the germinal centers of lymph nodes. Cul-5 is not expressed by normal human lung which is interesting given that Cul-5 was cloned from a human lung cancer cell line. It is concluded that Cul-5 is an intracellular protein that is expressed by several human cell types, and may play roles in skeletal muscle vascular physiology, kidney physiology, and immune cell function.

Introduction

The human vasopressin-activated calcium-mobilizing receptor (HVACM) was cloned from a human small cell lung carcinoma (SCLC) cell line (Longo, Fay et al. 1998), and has recently been renamed Cul-5. The Cul-5 cDNA (GenBank accession number AF017061) encodes a putative protein of 780 amino acids, with a predicted molecular weight of 91 kD. Cul-5 can interact specifically with vasopressin (AVP), which promotes intracellular calcium mobilization. However, Cul-5 is structurally distinct from the AVP V_{1a} receptor subtype: Cul-5 contains no predicted transmembrane domain (Longo, Fay et al. 1998). The V_{1a} receptor contains seven transmembrane domains, is G-protein-linked, and is also capable of promoting intracellular calcium mobilization in response to AVP stimulation through a mechanism that involves inositol triphosphate (Howl, Ismail et al. 1991; Nathanson, Moyer et al. 1992; Thibonnier, Auzan et al. 1994).

Cul-5 is homologous to the cullins, a relatively new family of proteins, which are involved in the ubiquitin-mediated degradation of cell cycle proteins (Kipreos, Lander et al. 1996; Byrd, Stankovic et al. 1997; Longo, Fay et al. 1998). The function of Cul-5 is unknown, although its considerable C-terminal homology to the cullins suggests that it too may be involved in ubiquitin-mediated cell cycle protein

degradation (Longo, Fay et al. 1998). The nuclear cullin protein Cdc53, which has orthologues expressed in yeast and humans, interacts specifically with the ubiquitin ligase Cdc34 and the F-box protein Cdc4 (Feldman, Correll et al. 1997). Together, these proteins direct the ubiquitination of the G1/S phase transition regulatory protein Sic1 (Mathias, Johnson et al. 1996; Verma, Feldman et al. 1997). Cdc53 has no catalytic activity, and is believed to act as a scaffolding protein for the assembly of the ubiquitin ligase complex (Patton, Willems et al. 1998). In this capacity, Cdc53 (and other cullins) may partially determine the substrate specificity, and hence the functional specificity, of ubiquitin ligase complexes (Kipreos, Lander et al. 1996; Willems, Lanker et al. 1996). The growing number of cullin family proteins in humans suggests that different cullins may be involved in different ubiquitination events (Kipreos, Lander et al. 1996). Therefore, an analysis of the tissue-specific expression of Cul-5 might provide clues as to the function of this protein in humans.

Cul-5-specific polyclonal antibodies were prepared by immunizing rabbits with a synthetic peptide representing the N-terminal 15 amino acids of Cul-5, conjugated to keyhole limpet hemocyanin. Analysis of Cul-5 expression in various AMeX-processed normal human tissues was evaluated by immunohistochemistry. The normal human tissues lung, kidney, liver, lymph node, and skeletal muscle were fixed immediately after biopsy by the AMeX method, which is thought to preserve the antigenicity of cellular proteins (Kiernan 1990).

Positive staining for Cul-5 was noted in capillary endothelial cells of skeletal muscle, cells comprising the distal convoluted tubules of the kidney, Kupffer cells inhabiting the liver sinusoids, and cells inhabiting the germinal centers of lymph nodes (which are presumably lymphocytes undergoing antigenic challenge and differentiation in mature lymphocytes).

Of interest was the apparent absence of staining of human lung epithelium, or lung capillary endothelium, but a positive staining of immune cell types such as polymorphonuclear (PMN) cells, occupying the alveolar spaces. Positive staining of PMNs in the lung was also noted with pre-immune antibodies, and when anti-Cul-5 antibodies were pre-incubated with the Cul-5 peptide immunogen. The staining of PMN cells was further investigated using flow cytometric analysis of isolated peripheral blood lymphocytes, monocytes, and PMNs. These experiments confirmed that PMN staining occurred with equal intensity using either the anti-Cul-5 antibodies, or pre-immune antibodies.

These results indicate that Cul-5 may be playing roles in the vascular physiology of skeletal muscle, as well as in the physiology of the distal convoluted tubules of the kidney. Furthermore, Cul-5 appears to be expressed in maturing lymphocytes of the lymph nodes, as well as in Kupffer cells, which are macrophages endogenous to the liver, suggesting that Cul-5 may influence myelopoiesis or immune cell function. Cul-5, [✓] which was cloned from human SCLC, was not observed in lung tissue, ^{and this} suggesting that SCLC does not arise from lung tissue proper, but rather from cells of myeloid origin (Ruff and Pert 1984). X

Materials and methods

Development of polyclonal antibodies directed against an Cul-5 epitope

Cul-5-specific polyclonal antibodies were prepared by and purchased from Bio-Synthesis, Inc. (Lewisville, TX). Two New Zealand White rabbits were immunized with a synthetic peptide of the following amino acid sequence:

MATSNLLKNKGSLQFC. (This sequence comprises the amino terminal 15 amino acids of the putative Cul-5 protein.) The peptide was conjugated to the carrier protein keyhole limpet hemocyanin via a sixteenth C-terminal cysteine residue on

the peptide. Rabbit serum antibody titers were assessed using an ELISA-based assay. When titers were satisfactory, the rabbits were sacrificed and their blood collected by cardiac puncture, and antibody-rich supernatant, or serum, was collected and stored at -20° C. For this study, antibodies in serum were purified using a protein A-Sepharose column (Ausubel, Brent et al. 1988). Specificity of the antibodies for Cul-5 was confirmed using Western blot analysis of cellular protein lysates from COS-7 cells over-expressing the cloned Cul-5.

Tissue Preparation

All normal tissues used in these studies were obtained from the Pathology Department at the Dartmouth Hitchcock Medical Center, Lebanon, NH, and represented the following tissues: lung, liver, kidney, lymph node, and skeletal muscle. All tissues were fixed by the AMeX (Acetone, Methyl benzoate, Xylene) method (Sato, Mukai et al. 1986). Following fixation, the tissues were embedded in paraffin wax, sliced into 4 μ m sections, and mounted on silane-coated glass slides. Tissues on slides were de-paraffinized by initially baking the slides in 60° C oven for 20 min, followed by four submersions in 100% Xylene, for 5 min each. The slides were then dipped in acetone 20 times, and washed with ddH₂O for 2 min. *ask Vivie*

Immunohistochemistry

Endogenous peroxidase activity was removed from de-paraffinized tissues by immersion in a 0.3% peroxide solution for 20 min, followed by washing with ddH₂O, and then PBS buffer. This was followed by washing with 95% ethanol, and rinsing with PBS buffer. Human Fc receptors were blocked by addition of 100 μ l of human IgG in PBS buffer (1 mg/ml), incubated for 20 min at room temperature, and then rinsed with PBS buffer. The primary antibodies (either anti-Cul-5 or pre-immune IgGs) were prepared in stock solutions of 1 mg/ml in PBS buffer

containing 0.1% sodium azide, and were utilized at dilutions of 1:400 in PBS buffer containing 10% goat serum, and the tissues were incubated for 12-18 h at 4°C. (Antibody dilutions were initially tested ranging from 1:50 to 1:1600. A dilution of 1:400 of protein A-purified antibodies was deemed optimal on the basis of contrast staining between anti-Cul-5 antibodies and pre-immune antibodies.) The following day the antibodies were removed by washing with PBS buffer at room temperature.

A solution of biotinylated secondary goat anti-rabbit antibody was applied to each section and incubated for 30 min. Following washing of the secondary antibody with PBS buffer ABC solution (avidin-biotin-horseradish peroxidase, Ventana Medical Systems, Tucson, AZ) was added to each section for 30 min. Non-bound peroxidase was removed by washing with PBS buffer. Visualization of staining was performed by addition of a 3,3' diaminobenzidine (DAB) for 8 min, rinsed with PBS buffer, counterstained with Hematoxylin, rinsed with PBS buffer, washed with xylene, and coverslipped with Aquamount.

Flow cytometric analysis of HVACM expression in PMNs

Peripheral blood cells (lymphocytes, monocytes, and PMNs) isolated using a Ficoll gradient, washed with PBS buffer containing 0.1% bovine albumin serum (BSA), Fraction V. Cells were stained for Cul-5 intracellularly. The cells were fixed in PBS buffer containing 1% PFA for 30 min, on ice, and then permeabilized with staining buffer (PBS buffer containing 1% BSA, 0.1% sodium azide, and 0.3% saponin). Anti Cul-5 antibodies or pre-immune antibodies were diluted in staining buffer (final concentration of antibodies: 0.4 µg/ml). The cells were incubated in the presence of antibodies for 15 min, on ice, washed with staining buffer, stained with goat anti-rabbit-FITC (Life Technologies, Gaithersburg, MD), and finally washed again with staining buffer. The cells were analyzed with a Becton Dickinson FACScan five parameter flow cytometer. Cul-5 expression was analyzed by laser-stimulated FITC

excitation (495 nm) and detected emission (519 nm). Expression of Cul-5 was gauged against pre-immune antibodies by comparison of histograms of fluorescence intensity.

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

Protein lysates from COS-7 cells over-expressing Cul-5 were separated by 10% SDS-PAGE at 75 V for 2.5 h at room temperature in a Tris-glycine buffer (25 mM Tris-base, pH 8.3, 192 mM glycine, 0.1% SDS) (Laemmli 1970; Ausubel, Brent et al. 1988). The gels were rinsed briefly with distilled water, and proteins were transferred to a methanol-activated Imobilon-P membrane (0.45 μ m porosity) (Millipore Inc., Bedford, MA) at 15 V, 18 h, at 4°C in a Tris-glycine buffer (20 mM Tris-base, pH 8.0, 15 mM glycine) containing 20% methanol (Towbin, Staehelin et al. 1979).

Immunoblotting with anti-Cul-5 antibodies

Following Western transfer, the Imobilon-P membranes were blocked in blocking buffer (10 mM Tris-HCl, pH 7.3, 100 mM NaCl, 0.1% Tween-20, 5% non-fat dried milk) for 1 h at room temperature. Cul-5 antibodies (10 μ g) were diluted in 5 ml of blocking buffer and added to the membrane for 15 min, followed by repeated washes with wash buffer (10 mM Tris-HCl, pH 7.3, 100 mM NaCl, 0.1% Tween-20). Specific Cul-5 antibodies were detected by incubation of the membrane with goat anti-rabbit-horse radish peroxidase-conjugated secondary antibody (Life Technologies, Gaithersburg, MD) diluted 1:20,000 in blocking buffer for 30 min at room temperature, repeated rinsing with wash buffer, and visualization using enhanced chemiluminescence (ECL) (Amersham, Piscataway, NJ).

Results

Characterization of Cul-5 antibodies

Anti-Cul-5 polyclonal antibodies detected two proteins of molecular sizes ~95 kD and ~50 kD in cell lysates of COS-7 cells over-expressing Cul-5 (Fig. 9) by SDS-PAGE and Western blot analysis. The pre-immune antibodies did not detect any proteins, indicating the specificity of the anti-Cul-5 antibodies. Pre-incubation of the anti-Cul-5 antibodies with the immunizing peptide successfully blocked the staining of these two bands (data not shown).

Kidney Cul-5 expression

Positive staining of this tissue appeared to be localized exclusively to the distal convoluted tubules (Fig. 1). This staining was intracellular, and no membrane staining, either apical or basolateral, was noted. No other tubular segments or regions of the kidney demonstrated positive staining for Cul-5.

Liver Cul-5 expression

Positive staining of liver was localized exclusively to cells inhabiting the liver sinusoids (Fig. 2). These cells were identified as Kupffer cells, macrophages that normally reside in the hepatic sinusoids. No other cells types within the liver demonstrated any staining for Cul-5.

Skeletal muscle Cul-5 expression

Positive staining of skeletal muscle was visualized in the endothelial cells comprising capillaries of this tissue (Fig. 3). This staining was intracellular, and was successfully blocked when anti-Cul-5 antibodies were incubated with tissues in the presence of a 10-fold molar excess of the immunizing peptide (data not shown). Some mild positive staining of the sarcomeres is also apparent.

Lymph node Cul-5 expression

Positive staining of the lymph nodes was localized to cells residing within the germinal centers of the nodes (Fig. 4). These cells, with their high cytoplasm-to-nucleus ratios, are most likely T- and B-lymphocytes undergoing antigenic challenge. No positive staining was evident in cells with little or no cytoplasm, located in the areas surrounding the germinal centers. These cells are presumably small lymphocytes that are resting in a pre-blast state, and have not yet undergone antigenic challenge.

Lung Cul-5 expression

Initial evaluation of lung tissue stained with anti-Cul-5 antibodies revealed no staining of the alveolar epithelium, or of endothelial cells comprising capillary vessels scattered throughout the tissue (Fig. 5). Pronounced positive staining of cells attached to the alveolar surface was observed. These cells were determined to be polymorphonuclear (PMN) cells, based on the multi-lobulated with no nuclear staining. Staining appeared to be cytoplasmic, and excluded from the nuclei. In some cases, staining of subcellular granules was noted. However, both the pre-immune controls (Fig. 6) and antigen-blocked controls (Fig. 7) also stained normal lung tissue in a similar fashion, indicating that staining of these cells was most likely an artifact due to either endogenous peroxidase activity that could not be successfully removed during tissue treatment with peroxide, or a failure to properly block F_c receptors located on these cells.

Flow cytometric analysis of Peripheral Blood Cells

Both anti-Cul-5 antibodies and pre-immune antibodies stained PMN cells with equal fluorescence intensity in flow cytometry performed on PMNs, confirming an

absence of specific Cul-5 antibody/Cul-5 protein interactions, and confirming the false-positive results from immunohistochemistry of the lung (Fig. 9).

Discussion

Immunohistochemical localization of Cul-5 to the distal convoluted tubule in human kidney corroborates evidence found for the rabbit VACM-1 receptor, which was cloned from, and immunolocalized to cells comprising the rabbit kidney distal convoluted tubule (Burnatowska-Hledin, Spielman et al. 1995). The significance of Cul-5 expression in the distal convoluted tubule is not clear, since no explicit function has yet been attributed to this protein (Byrd, Stankovic et al. 1997; Longo, North et al. 1997). However, results obtained in the current studies differ from those obtained for the rabbit: VACM-1 staining was reported to be basolateral (Burnatowska-Hledin, Spielman et al. 1995), while Cul-5 seems to be expressed intracellularly, with no apical or basolateral localization. Possible explanations for this apparent difference between VACM-1 and Cul-5 localization may be the tissue preparation method employed, or observational variance. VACM-1 immunohistochemistry in rabbit kidneys was performed on frozen tissue sections using immunofluorescence, while the studies reported here were performed on AMeX-processed tissues. The possibility that AMeX fixation altered the cellular localization of Cul-5, however minute, cannot be ruled out.

The staining of Kupffer cells within hepatic sinusoids raises the intriguing possibility that Cul-5 may be involved in the activities of liver macrophages. The hypothesized role of Cul-5 in ubiquitin-mediated degradation of cellular proteins, and the lability of Kupffer cells to small vasoactive peptides (Alexander 1998), begs the question of whether or not Cul-5, and AVP, have cell-specific function in Kupffer cells. Kupffer cells are modified macrophages that originate in the bone

marrow, and migrate to the sinusoids of the liver, where they differentiate into their final phenotype (Bouwens 1988; Naito, Hasegawa et al. 1997). Kupffer cells carry out typical macrophage duties like the clearance of IgA- and IgG-bacterial complexes (Bogers, Stad et al. 1992), as their macrophage phenotype would suggest, but also participate in several pathophysiologic processes of the liver (Cerra, West et al. 1988). During bacterial sepsis (endotoxic shock), ischemia, and injury, Kupffer cells synthesize and release the free radical nitric oxide (NO) (Alexander 1998). At low levels, NO is both bactericidal, as well as tumoricidal (Evans 1995; Alexander 1998). However, at higher levels, NO is toxic to the liver (Evans 1995). Prolonged stimulation or hyper-stimulation of Kupffer cells can lead to free-radical induced destruction of the liver (Cerra, West et al. 1988), despite the cytoprotective effects of antioxidants secreted by the endothelial cells lining the liver sinusoids (Moro, Jacoulet et al. 1994; Spolarics 1998). Liver necrosis in response to alcohol abuse is the indirect result of alcohol-induced release of endotoxins from bacteria in the gut, which stimulate the production and release of liver-damaging NO (as well as eicosanoids and tumor necrosis factor-alpha) from Kupffer cells (Thurman, Bradford et al. 1997). Conversely, Kupffer cells have profound effects on liver regeneration through their production of the hormone Hepatocyte Growth Factor (HGF) (Matsumoto and Nakamura 1991), and in response to pro-inflammatory cytokines (Diehl and Rai 1996). Kupffer cells also are a major producer of metalloproteinases, which may be important in liver restructuring following injury or necrosis, or which could possibly induce necrosis in some pathophysiological states (Arthur 1994). Whether or not Cul-5 plays a role in any of these processes is unknown.

The vasopressin receptor subtype V_{1a} is expressed in liver hepatocytes, and AVP-stimulation of these cells increases glycogen phosphorylase activity (Diehl and Rai 1996). The role of AVP in the hepatocyte function of humans appears to be

relatively minor when compared to that of the rat. In rat liver, AVP stimulates profound increases in glycogen phosphorylase activity, as well as in DNA synthesis (Howl, Ismail et al. 1991). AVP can evoke increases in $[Ca^{2+}]_i$ in hepatocytes located in the pericentral regions of the liver, and these Ca^{2+} "waves" propagate out into the periportal regions of the liver (Nathanson, Burgstahler et al. 1995). The ability of VACM-1 (and by inference Cul-5) to evoke increases of $[Ca^{2+}]_i$ in response to AVP, in conjunction with the V_{1a} receptor's established role in hepatic Ca^{2+} homeostasis, paints a more complex picture of how AVP may be affecting liver Ca^{2+} metabolism and function (Burnatowska-Hledin, Spielman et al. 1995; Longo, Fay et al. 1998).

The selective positive staining for Cul-5 found in the germinal center of lymph nodes suggests that Cul-5 may play a role in the maturation or function of lymphocytes or macrophages that occupy the germinal centers. Several lines of evidence suggest that AVP can modulate cell-mediated immunity, and can influence the maturation of several immune cell types, and that these effects are mediated by several AVP receptor subtypes (Bell, Adler et al. 1992). AVP is released from the posterior pituitary in response to different forms of immune stress, in addition to its release in response to low osmolality or fluid loss (Bell, Adler et al. 1992). Macrophages express AVP receptors, and AVP stimulates cAMP production in these cells (Bell, Adler et al. 1993). Induction of interferon-gamma (IFN γ) production in mouse spleen cells through a V_1 -type cGMP-dependent mechanism has also been demonstrated (Johnson, Farrar et al. 1982; Johnson and Torres 1985; Elands, Resink et al. 1990). Vasopressin V_1 receptor agonists and antagonists were able to stimulate and block, respectively, IFN γ production in splenic lymphocytes, while vasopressin V_2 receptor agonists and antagonists had no effect (Johnson and Torres 1988; Torres and Johnson 1988). AVP stimulates peripheral PMNs and B-cells to produce β -endorphin (Kavelaars, Ballieux et al. 1989). Several immune cell types, ranging from splenic plasma cells and immature lymphocytes, to immune

support cells like the thymic nurse cells (which are responsible for controlling the development and differentiation of immature T-cells within the thymus) are also influenced by AVP (Hammer and Skagen 1986; Liard 1986; Wiles, Grant et al. 1986). In its capacity as an AVP-binding protein, Cul-5 may be involved in some of the AVP-responsive physiologic phenomena observed in immune cells (Longo, Fay et al. 1998).

Endothelial cells comprising the capillary walls of skeletal muscle express Cul-5. No positive staining of capillary endothelial cells was noted in any of the other tissues studied, indicating that Cul-5 localization to capillaries was specific to the endothelial cells of skeletal muscle alone. AVP can reduce blood flow in skeletal muscle, as well as in skin (Hammer and Skagen 1986; Liard 1986; Wiles, Grant et al. 1986). Whether these effects are mediated through the classical AVP receptors, or through Cul-5, is unclear, since none of these studies tested the effects V₁ or V₂ receptor agonists or antagonists on blood flow (Hammer and Skagen 1986; Liard 1986; Wiles, Grant et al. 1986).

More intriguing is the role of AVP in the morphogenic differentiation of skeletal muscle cells. Cultures of L6 myoblasts, treated with 1 μ M AVP demonstrated an increase in myotube size, without cell division (Nervi, Benedetti et al. 1995). This effect was blocked by treatment of cells with AVP in the presence of a V₁ receptor antagonist (Nervi, Benedetti et al. 1995). AVP-induced expression of the muscle-specific transcription factor Myf-5 and the muscle-specific protein myogenin was also noted (Nervi, Benedetti et al. 1995). AVP-induced skeletal muscle differentiation may be mediated through the stimulation of phospholipase D (Naro, Donchenko et al. 1997). Given its proposed role as a cullin, Cul-5 may be partly responsible for catabolic processes (i.e. protein degradation) that are essential for AVP-induced skeletal muscle growth and differentiation.

Initial immunohistochemical evaluation of normal human lung indicated that Cul-5 was expressed in cells attached to the alveolar surface, but not in lung epithelial cells, or in lung capillary endothelial cells. Cul-5-expressing cells were evaluated to be PMNs, based on their multi-lobulated nuclear morphology. However, positive staining was also apparent in both the pre-immune controls and peptide controls employed in immunohistochemistry, indicating that staining of these PMNs was probably non-specific. This non-specific staining for Cul-5 was confirmed by flow cytometry on peripheral blood cells (PMNs, macrophages, and lymphocytes) stained with anti-Cul-5 antibodies. Evaluation of Cul-5 expression by Western Analysis confirmed that Cul-5 protein was absent from peripheral blood cells. This raises an interesting question: if neither lung epithelial cells or endothelial cells, nor immune cells inhabiting the alveolar spaces of the lung, express HVACM, then why is this protein expressed in an SCLC cell line (Longo, Fay et al. 1998)? (Conversely, why do Kupffer cells express Cul-5 protein, while their systemic and alveolar counterparts do not?) A transformed lung PMN might abnormally express this protein, whereas the precursor, untransformed cell, might not. The abnormal expression of Cul-5 in these tumors may be explained by the high frequency of chromosomal abnormalities found in the Cul-5 locus on human chromosome 11q22-23 in lung and breast tumors (Carter, Negrini et al. 1994; Rasio, Negrini et al. 1995; Tomlinson, Strickland et al. 1995; Kerangueven, Eisinger et al. 1997). It is also possible that this particular lung tumor did not originate in the lung at all, but rather from a transformed cell type that migrated to the lung. Since Cul-5 appears to be expressed in activated monocytes and lymphocytes in lymph nodes, it is possible that the SCLC cell line NCI-H146 originated from either alveolar macrophages or lymphocytes, or transformed macrophages and lymphocytes that migrated to the lung (Ruff and Pert 1984; Ruff and Pert 1987). Lung tumors, in particular SCLC tumors, may originate from alveolar macrophages: several SCLC

tumors exhibit both neuroendocrine and immune cell features, traits that are entirely consistent with the cell line NCI-H146 (Koros, Bey et al. ; Ruff and Pert 1984; North 1991). Moreover, alveolar macrophages become cytochemically altered in smokers and SCLC patients (Berman and Goldman 1992), suggesting that smoke-induced differentiation of macrophages, cells which are normally capable of movement between tissues by extravasation/intravasation, might produce tumor cells with an inherent ability to "metastasize."

FIGURE LEGENDS

Figs. 1-4. Positive Cul-5 intracellular immunostaining of distal convoluted tubule (D) cells (Fig. 1), Kupffer cells (K) in hepatic sinusoids (Fig. 2), capillary endothelial cells (C) in skeletal muscle (Fig. 3), and lymphocytes (L) inhabiting the germinal centers of lymph node (Fig. 4). No positive immunostaining was noted with pre-immune antibody controls, or antigen-blocked antibody controls, for the above tissues. D, distal convoluted tubule; K, Kupffer cells; C, capillary endothelial cells; L, lymphocytes.

Figs. 5-7. Positive Cul-5 intracellular immunostaining of immune cells in the alveolar spaces of normal human lung (Fig. 5). Positive staining was also observed in pre-immune control tissue (Fig. 6), and antigen-blocked antibody control tissue (Fig. 7), suggesting that anti-Cul-5 antibodies were non-specifically interacting with these cells.

Fig. 8. Flow cytometric histograms of peripheral blood immune cells (monocytes, lymphocytes, and polymorphonuclear cells) stained with anti-Cul-5 antibodies (A) and pre-immune antibodies (B). The similar flow cytometric staining profiles with these two antibodies corroborate the non-specific staining noted in immunohistochemistry of the lung (Figs. 5-7).

Fig. 9. Characterization of Cul-5 rabbit polyclonal antibodies. Cell lysates from COS-7 cells over-expressing Cul-5 were subjected to SDS-PAGE and Western blotting, and immunoblotted with either pre-immune antibodies (PI) or anti-Cul-5 antibodies (α Cul-5), and a secondary anti-rabbit-HRP antibody. Two protein bands of approximate molecular sizes 95 kD and 50 kD were visualized using anti-Cul-5 antibodies. These two proteins were not detected with the pre-immune control lane

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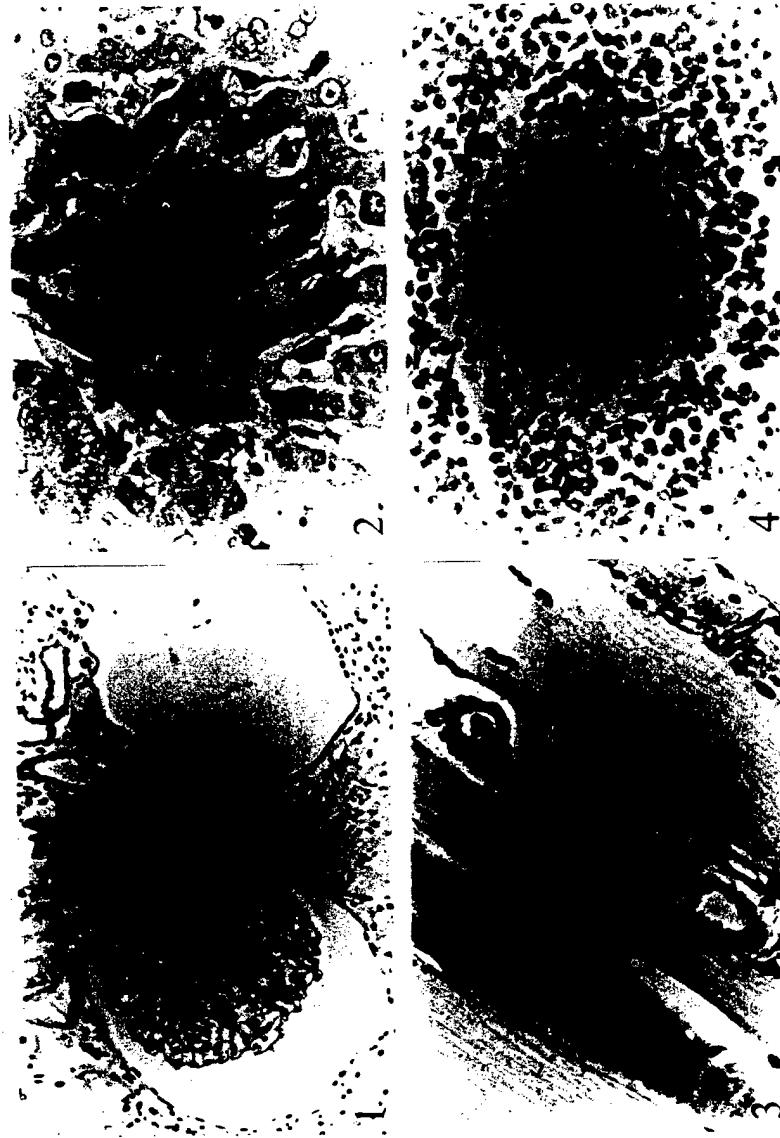
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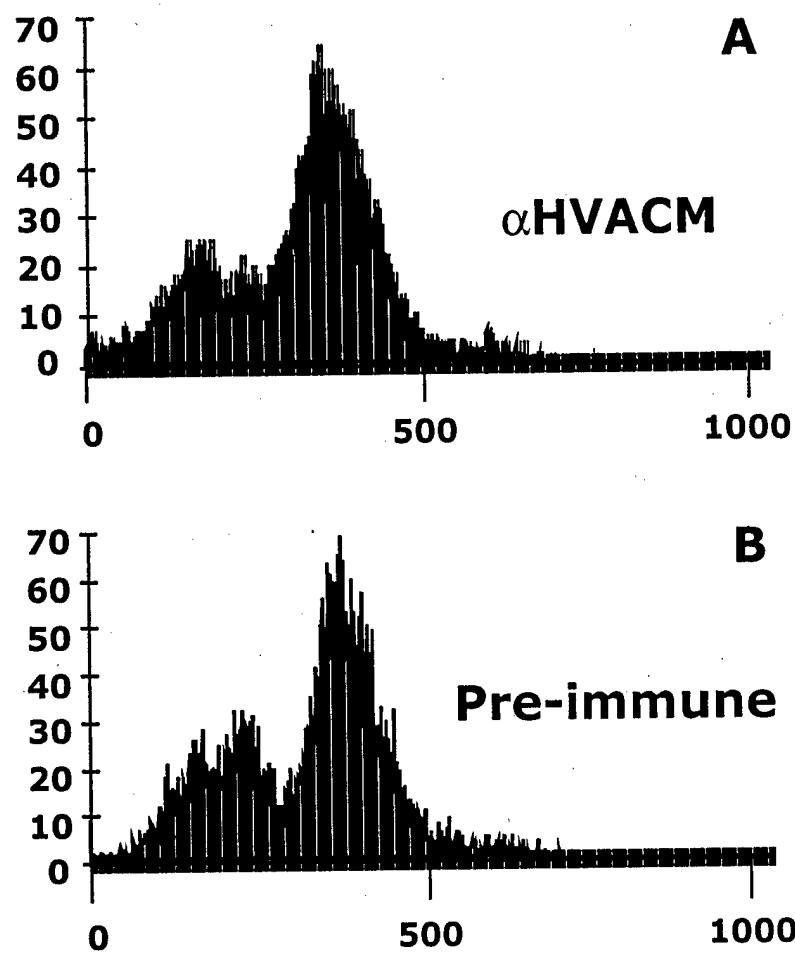
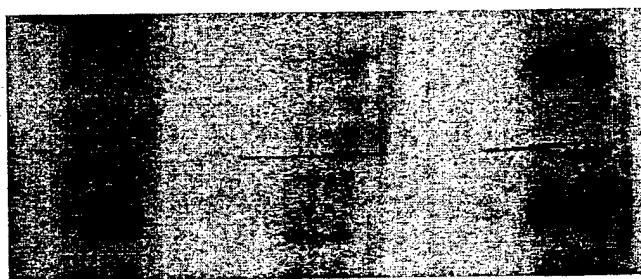


Figure 8

2° PI α H



← **95 kD**

← **50 kD**

Figure 9

Submitted to Cancer Research

TITLE

Small cell lung cancer expresses a functional form of VACM-1, a putative vasopressin receptor and cullin family member.*

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SUMMARY

Vasopressin (AVP)¹ may be involved in human lung cancer pathophysiology, as an autocrine/paracrine hormone. AVP can act through four classes of receptors: V₂, V_{1a}, V_{1b}, and the vasopressin-activated calcium mobilizing (VACM-1) receptor, a structurally unique member of this group. (Recently, a highly homologous cDNA, termed Hs-VACM-1, was cloned from human placental mRNA.) A human orthologue of rabbit VACM-1 is expressed in the small cell lung cancer cell line NCI-H146. AVP induced an increase in intracellular free calcium in this cell line, but no increase in total inositol phosphates. NCI-H146 expressed three distinct mRNA bands of 3.5, 5 and 6.5 kilobases by Northern blot. RT-PCR products of the predicted sizes of 674 bp and 193 bp were amplified from NCI H-146, normal human lung and kidney RNA, using primers designed from the rabbit VACM-1 and a human expressed sequenced tag with homology to VACM-1. Overlapping 5'RACE and 3'RACE products were cloned from NCI-H146 RNA, and sequenced. The clone, named HVACM, encodes an open reading frame (ORF) of 780 amino acids and shares a high degree of amino acid identity to the rabbit VACM-1 (97%) and the Hs-VACM-1 (97%) as well as the cullins, a family of proteins involved in ubiquitin-mediated cell cycle regulation.

INTRODUCTION

Several human arginine vasopressin (AVP) receptors of the seven-transmembrane domain, G-protein linked, class have been cloned and characterized (1, 2, 3). The V₂ receptor is coupled to the enzyme adenylate cyclase. The expression of this receptor has been demonstrated in the kidney, and its activation by AVP leads to the insertion of aquaporins in the luminal membranes of principal cells, and increased water reabsorption (4, 5, 6, 7, 8, 9). Two classes of V₁ receptor have been defined. The V_{1a} receptor is expressed in arterial smooth muscle, and activates phospholipase C, which in turn leads to the generation of inositol triphosphate (IP₃) and diacylglycerol (DAG), and elevates intracellular calcium and activates protein kinase C, respectively (10, 11, 12, 13, 14, 15, 16). Activation of this receptor is responsible for contraction of arterial smooth muscle, mediating pressor activity and maintenance of blood pressure. The V_{1b} receptor also known as the V₃ receptor (17), is expressed in the adenohypophysis (18, 19). The V_{1b} receptor can also stimulate phosphoinositide hydrolysis and increases in intracellular calcium; several members of the phospholipase family have been hypothesized as partners for this receptor (18, 19, 20).

Recently, a potential new member of the AVP receptor family was expression-cloned from rabbit kidney medulla. This putative vasopressin activated calcium mobilizing receptor, VACM-1 (GenBank accession number S78157), encodes a protein larger than the serpentine receptors (780 amino acids), and does not fit the seven-transmembrane domain model (21). VACM-1 has been postulated to be a single-transmembrane domain protein due to the presence of a hydrophobic segment of 20 amino acids, though its topology or orientation with the membrane have not been definitively determined. VACM-1 overlaps mechanistically with the V₁ receptors: the stimulation of these receptors induces increases in intracellular calcium (21, 22). In contrast to the V₁ receptors, VACM-1 is not G-protein linked, and can generate Ca²⁺ increases without the production of IP₃. The mechanism for the Ca²⁺ elevation induced by this receptor is unknown.

A human homologue of this receptor, Hs-VACM-1 (GenBank accession number

X81882), was recently cloned and mapped to chromosome 11 (q22-23) (23). This discovery was made through a study of the disorder Ataxia Telangiectasia, which maps to the same locus (24). The receptor mRNA is ubiquitously expressed (23), but the physiology or function of this protein is unknown. The homology of this receptor with the cullins, a family of proteins implicated in the ubiquitin-mediated degradation of cyclins (or cyclin/CDK inhibitors) suggests a role for the VACM proteins in similar degradative processes (25, 26, 27, 28).

Work in our laboratory has demonstrated the presence of a VACM-1-like mRNA in the small cell lung cancer cell line NCI-H146, and a preliminary report on this work has been presented (29). We have performed Northern blot analysis for the mRNA, and have isolated a cDNA clone for this putative receptor, that we have named the human vasopressin-activated calcium mobilizing (HVACM) receptor. HVACM has considerable homology to both the genomic human (HS-VACM-1) and the rabbit (VACM-1) receptors, and contains an ORF encoding a putative protein of 780 amino acids. HVACM could be a functional protein in NCI-H146, since this cell line does not appear to express mRNAs for any of the other known AVP receptors (V_2 , V_{1a} , and V_{1b}), but responds to AVP stimulation with an increase in intracellular calcium originating from an intracellular store that is distinct from the ryanodine-sensitive smooth endoplasmic reticulum calcium reservoirs.

EXPERIMENTAL PROCEDURES:

Cell culture - NCI-H146 cells were obtained from the American Type Culture Collection (Rockville, MD), and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan UT), without antibiotics.

RT-PCR analysis of HVACM mRNA expression - Total RNA from NCI-H146 was analyzed for the presence of VACM-1-like mRNA using the reverse transcription polymerase chain reaction (RT-PCR). Briefly, 1 ug of total RNA was reverse transcribed using the Superscript II enzyme (Life Technologies, Gaithersburg, MD), and subsequently subjected to 35 cycles of PCR using the *Taq* polymerase enzyme (Perkin Elmer, Branchburg, NJ) (28).

Synthetic primers 1432F (5' GAATGGCTAAGAGAAGTTGGTATG 3'), 2082R (5' TCTTCTCTCATCCTTCTGTAGTG 3'), 325F (5' CATAACCAACTTCTCTTAGCCACTC 3'), and 156R (5' CACCATTAAGCAAAACTACCTCTG 3') were designed using the program Oligo™ for the Macintosh, and purchased from Life Technologies (Gaithersburg, MD). The primers 1432F/2082R and 325F/156R amplify a 674 base pair (bp) fragment and a 193 bp fragment, respectively, within the putative ORF for VACM-1. Conditions for PCR involved an initial denaturation step (96 °C, 2.5 min). This was followed by 35 cycles of the following sequence: 96 °C for 45 s; 60 °C for 45 s; and 72 °C for 1.5 min, and a final extension time of 10 min. RT-PCR products were separated by 1% agarose gel electrophoresis, and visualized with ethidium bromide staining over a UV transilluminator.

Northern blotting of HVACM mRNA - Total RNA was extracted from NCI-H146 using 4 M guanidinium thiocyanate and subsequent cesium chloride gradient centrifugation, and electrophoresed through a 1.2% agarose gel containing 1.1% formaldehyde. The RNA was transferred to nitrocellulose overnight using 20xSSC as the mobile phase, followed by baking of the membrane (80 °C, 2 h). The membrane was then incubated in 50% formamide, 5 x SSPE, 5 x Denhardt's reagent, 0.1 mg/ml salmon sperm DNA, and 0.1% SDS, at 42 °C for 2 h. This was followed by incubation in the presence of a radiolabeled probe for 16 h at 42 °C. The probe corresponds to the 674 bp fragment generated by RT-PCR from NCI-H146 total RNA using the primer pair 1432/2082, and was ³²P-labeled with a DecaPrime DNA labeling kit (Ambion, Austin, TX). Any non-specifically bound probe was removed from the membrane by washing for 20 m at RT with 10 ml 1 x SSC, 0.1% SDS. Three additional washes were performed at 68 °C with 0.2 x SSC, 0.1% SDS. Interaction with the probe was resolved by autoradiography (30).

Isolation of HVACM nucleotide clones - Both 5'- and 3'-rapid amplification of cDNA ends (RACE) (25, 31) were employed to clone the open reading frame of this gene. An initial reverse transcription was performed (see above) using the primer 138R (5' TTGTTTTGTAAGGTAAGGCAGAG 3'). 5'RACE was performed using the primers 5'ATGF (5' TCCAAGTTAAAGAACATGGCG 3') and 2082R. 3'RACE was performed using the

bovine serum albumin for 24 h at 37 °C. The cells were assayed for total inositol phosphate production in a physiological salt solution containing lithium chloride (PSS) pH 7.35, with the following composition: NaCl (105 mM), LiCl (30 mM), NaHCO₃ (4.2 mM), KCl (5.9 mM), CaCl₂ (1.8 mM), MgCl₂ (1.4 mM), NaH₂PO₄ (1.2 mM), glucose (11 mM), HEPES (10 mM). Following three washes with 5 ml PSS, the cells were incubated in the same solution for 10 m at 37 °C. They were stimulated with 1000 nM AVP for a 30 min time period at 37 °C. Aliquots were periodically removed and total inositol phosphates extracted. For this extraction, the cell suspension was added to one tenth (1/10) volume (v/v) of 5 N perchloric acid, 1 mM EDTA, and 5 mM DEPTA, and placed on ice for 30 min. Subsequently, one volume (v/v) of 1.5% K₂CO₃ was added to the acid extracts to precipitate cellular debris, and the extracts were placed on ice for 2 h. After centrifugation, neutralized extracts of 1 ml were loaded onto AG1-X8 resin (formate form) (BioRad, Hercules, CA) columns equilibrated with 20 mM NH₄OH. The columns were washed sequentially with 4 ml 40 mM NH₄OH, 4 ml of 40 mM ammonium formate, and finally with 4 ml 2 M ammonium formate/0.1 M formic acid. For detection, 1 ml of the final wash was added to 10 ml of Hydrofluor scintillation fluid, and values of counts per minute (cpm) from [³H]phosphoinositides were obtained. Data were obtained from five independent experiments and expressed as an average ratio of the control values, and their standard error.

RESULTS:

RT-PCR analysis of HVACM RNA - Bands of the predicted sizes 674 bp and 193 bp, were generated using the VACM-1 selected primers 1432F/2082R and 325F/156R, indicating that an mRNA with considerable homology to the rabbit VACM-1 was being expressed in NCI-H146 (Figure 1). Bands of similar size were also amplified from normal human kidney and normal human lung RNA (data not shown).

Northern Analysis of HVACM RNA - Messenger RNAs of three distinct sizes were observed upon Northern analysis of total NCI-H146 RNA (Figure 2). These sizes were 6.5, 5.0,

primers 1432F and 138R. The *Pfu* we polymerase (Stratagene, La Jolla, CA) was utilized for higher fidelity PCR. After an initial denaturation step (96 °C, 2.5 m), 35 cycles of the following sequence were conducted: 96 °C for 45 s; 55 °C for 45 s; and 72 °C for 8 min. These cycles were followed with a final extension time of 20 min. PCR products were visualized with gel electrophoresis and ethidium bromide staining/UV illumination, and cloned into the pZERO-(Blunt) vector (Invitrogen, Portland, OR) for further analysis.

DNA sequencing of HVACM RACE clones - All sequencing was performed using the ABI/Prism automated sequencing system (Perkin Elmer, Branchburg, NJ). The software packages Align and MegAlign (DNAStar, Inc.) and Gene Inspector (Textco, Inc.) were employed for sequence analysis. Protein motifs were identified using a program available on the Internet, MotifFinder (Institute for Chemical Research, Kyoto University). Hydrophobicity analysis of protein was performed using the algorithm of Kyte and Doolittle (32).

Calcium analysis - Cells (2.0×10^6 cells/ml) were incubated in serum-free media in the presence of the calcium indicator Fura-2AM (final concentration 5 mM) (Calbiochem, San Diego, CA), at 37 °C for 1 h. The cells were washed and resuspended in Dulbecco's PBS with 2.5 mM Ca^{2+} or without Ca^{2+} at a final concentration of 10^6 cells/ml. The cell suspension was pre-warmed (to 37 °C) and analyzed in a quartz cuvette. $[\text{Arg}^8]\text{AVP}$, the V_{1a} agonist $[\text{Phe}^2, \text{Ile}^3, \text{Orn}^8]\text{AVP}$, or the V_{1b} agonist $[\text{deamino}^1\text{D}-3-(\text{pyridyl})\text{Ala}^2\text{Arg}^8]\text{AVP}$ were added directly to the cuvette. All peptides were purchased from Bachem (Torrance, CA). When indicated, cells were pre-incubated with the SER Ca^{2+} blocker TMB-8 (final concentration 10 μM) for 10 min at 37°C. Using a Perkin Elmer LS50B luminescence spectrometer, the cells were subjected to dual excitation wavelengths of 340 and 380 nm. The ratio of emission intensity at 485 nm with each of these excitation wavelengths (I/I) was determined. (The resulting I/I value is an arbitrary indicator of increases in $[\text{Ca}^{2+}]_i$.)

Inositol Phosphate analysis of NCI-H146 - Total inositol phosphates were quantified using a modification of the method of Berridge et al. (33). NCI-H146 cells were labeled with [^3H]myo-inositol (DuPont-NEN, Boston, MA) in myo-inositol-free RPMI 1640, with 0.1%

and 3.5 kilobases (kb). This result was in agreement with other published results (23).

Isolation of an ORF clone of HVACM from NCI-H146 - Overlapping 5'- and 3'-RACE
products of ~2.1 kb and 1.1 kb were amplified, cloned in the vector pZERO-Blunt (Invitrogen), and sequenced. This clone contains a ORF of 2340 nucleotides, encoding a putative protein of 780 amino acids (Figure 3). The HVACM protein shares 97% identity with both Hs-VACM-1 and VACM-1 through comparisons using BLAST. Neither Gene Inspector™ nor MOTIF predicted an amino acid sequence with sufficient hydrophobicity to classify it as a transmembrane protein (Figure 4). No signal sequence was detected in this protein. However, several other unconfirmed motifs were predicted. The ORF of HVACM contains two protein kinase A (PKA) phosphorylation domains at Thr427 and Ser731. Additionally, there are 15 casein kinase II phosphorylation sites and 15 PKC phosphorylation sites. A single tyrosine kinase phosphorylation domain resides at Tyr207. There are two myristylation sites contained by residues 180..185 and by 664..669. There are three N-glycosylation sites represented by residues Asn145, Asn289, and Asn566. Finally, a cullin homology domain was identified in the C-terminal 27 amino acids of HVACM. Additional homology between HVACM and the cullins outside of this region was also detected.

AVP does not stimulate the production of phosphoinositides in NCI-H146 - An increase in total phosphoinositides (IP) was not observed following treatment with 1000 nM AVP (Figure 5). A slight decrease in total IP levels was actually apparent in the first 10 minutes following AVP treatment indicating that an IP₃-dependent mechanism was not responsible for the AVP-induced increases in intracellular calcium.

AVP modulates intracellular Ca²⁺ in NCI-H146 - An increase in [Ca²⁺]_i was observed in NCI-H146 after treatment with 1000 nM AVP (Figure 6a). Neither a V1a agonist (3 uM) or a V1b agonist (36 uM) were able to elicit [Ca²⁺]_i release when used at concentrations with similar activity to [Arg⁸]AVP (Figures 6b and 6c, respectively) (34, 35, 36). Depletion of Ca²⁺ from the medium did not block the action of AVP (Figure 6d), nor did pre-treatment of the cells with the SER-specific Ca²⁺ blocker TMB-8 (Figure 6e).

DISCUSSION:

The cDNA that we have named HVACM shares considerable amino acid identity with both Hs-VACM-1 and the rabbit VACM-1. In all likelihood, all three forms probably encode proteins sharing identical mechanistic and physiologic properties. The considerable sequence similarity between the human and rabbit proteins suggests a highly conserved evolutionary role for this protein.

The interaction of AVP with VACM-1 is able to induce increases in intracellular calcium. In this study, the inability of TMB-8 to block intracellular release of calcium suggests that HVACM (and therefore VACM-1) is stimulating an as yet undefined intracellular pool of calcium ions.

In addition to the basolateral localization of VACM-1 in rabbit kidney collecting duct, there was also a prominent cytoplasmic localization, and a minor nuclear localization, of this protein as evidenced from interactions with an antibody to its C-terminal domain in fluorescence microscopy (21). While this has yet to be confirmed for HVACM in the cell line NCI-H146, it raises the distinct possibility that VACM-1 (and HVACM) are not transmembrane proteins, but rather cytoplasmic proteins that associate with the plasma membrane weakly and/or dynamically. The position of HVACM in different cellular compartments or plasma membranes may be mediated through its association(s) with other proteins. AVP, by virtue of its interaction with HVACM, may control the localization and/or function of HVACM. The myristylation and glycosylation domains on the proteins may allow VACM-1 and HVACM to associate with the plasma membrane. Clearly, more study is required to understand the cellular localization of VACM proteins.

Since mRNAs for VACM proteins are co-expressed in many of the same tissues as the V_{1a} receptor, the important question of functional overlap is raised. Are these receptors functionally synergistic and involved in the same physiologic processes? Are they antagonistic and involved in each other's regulation? Do they have distinct physiologic roles and exist outside

of the other's regulation?

Certainly, the (unconfirmed) presence of copious PKC domains in HVACM insinuates that some inter-regulation between HVACM and V1a may occur physiologically. Future experiments should determine whether or not AVP can stimulate the phosphorylation of HVACM in cell systems where V1a and HVACM are co-expressed. Additionally, the presence of two PKA domains in HVACM suggests some form of inter-regulation with the V2 receptor may exist, and should be investigated.

Perhaps the most intriguing aspect of HVACM is its considerable homology to the cullins, a family of proteins implicated in the ubiquitin-mediated degradation of G1- and M-phase cyclins, or their inhibitors (37, 38). The cullins appear to serve a highly conserved regulatory role in the cell cycle of eukaryotes from yeast (37) to humans (36). By virtue of this domain, HVACM may be involved in the ubiquitin-mediated degradation of cellular proteins.

Individual cullin proteins are a part of defined multimeric protein complexes that serve as functional ubiquitin ligases (25, 26, 27, 28). The interaction of cullins with these complexes is mediated through the C-terminal amino acids that form the cullin homology domain (25). HVACM may also associate with other proteins as a necessary prerequisite for its functions in this enzymatic capacity. Whether cullins provide substrate specificity or enzymatic activity to these ubiquitin ligase complexes, or are merely scaffold proteins for these complexes, is not clearly understood (26). The fact that several cullin subtypes exist in humans suggests that the cullin proteins may serve tissue- or hormone-specific regulatory roles (39).

The ability of VACM-1 to specifically bind to AVP suggests that this hormone may affect the function of VACM-1/HVACM. The potential regulatory role of AVP in the physiology and function of HVACM warrants further investigation.

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FOOTNOTES

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The nucleotide sequence of HVACM cloned from NCI-H146 has been deposited in the GenBank database under GenBank Accession Number AF016071.

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ABBREVIATIONS

¹The abbreviations used are: AVP, [Arg8]vasopressin; PBS, phosphate buffered saline; IP, phosphoinositides; IP₃, inositol triphosphate; [Ca²⁺]_i, intracellular calcium; RT-PCR, reverse transcription polymerase chain reaction; PKA, protein kinase A; PKC, protein kinase C; ORF, open reading frame; TMB-8, 8-(Diethylamino)-octyl-3,4,5-trimethoxybenzoate, HCl; SER, smooth endoplasmic reticulum.

FIGURE LEGENDS

FIG. 1. RT-PCR for VACM-1 from NCI-H146 total RNA. Primers use for PCR: lane 1; 1432/2082, lane 2; 156/325.

FIG. 2. Northern blot analysis of VACM-1 messenger RNA from NCI-H146 total RNA (20 μ g).

FIG. 3. Predicted open reading frame nucleotide sequence of HVACM cloned from NCI-H146. The putative amino acid sequence is also shown.

FIG. 4. Hydrophobicity profile of HVACM. Protein hydrophobicity was calculated using the algorithm of Kyte and Doolittle (32). The vertical axis represents hydrophobicity for HVACM amino acids, which are represented by the horizontal axis.

FIG. 5. Total inositol phosphate analysis of NCI-H146 following stimulation with 1000nM AVP over 30 minutes. Data are presented as the ratio of control (unstimulated) values of [3H]inositol counts per minute, +/- S.E. (n=5).

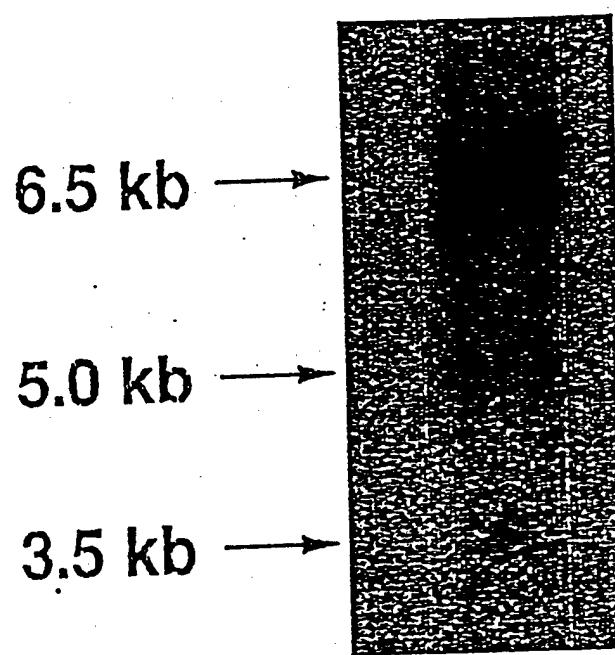
FIG. 6. Intracellular calcium analysis of FURA-2AM-loaded NCI-H146 cells. Stimulation with (a) 1000nM AVP, (b) V1a agonist, (c) V1b agonist, (d) 1000nM AVP minus extracellular calcium, (e) 1000nM AVP plus pre-incubation with 100uM TMB-8.

1 2

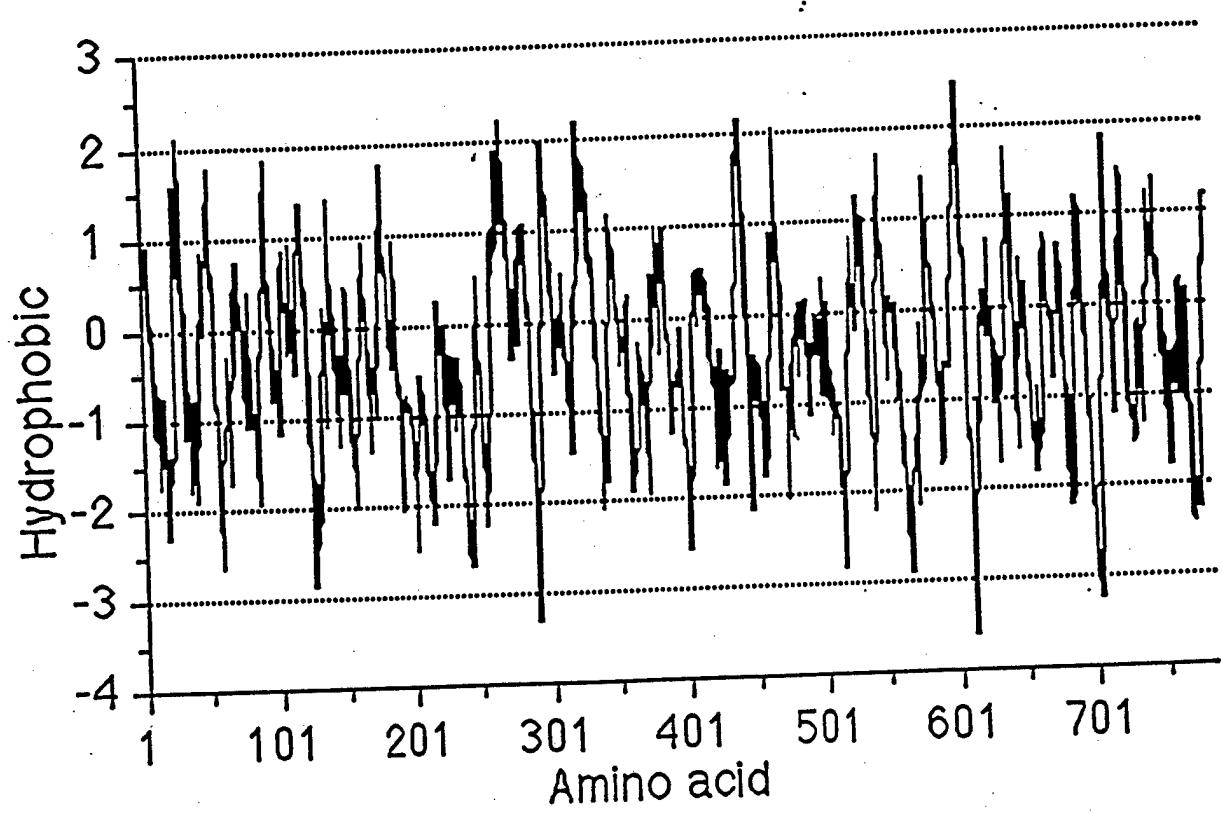
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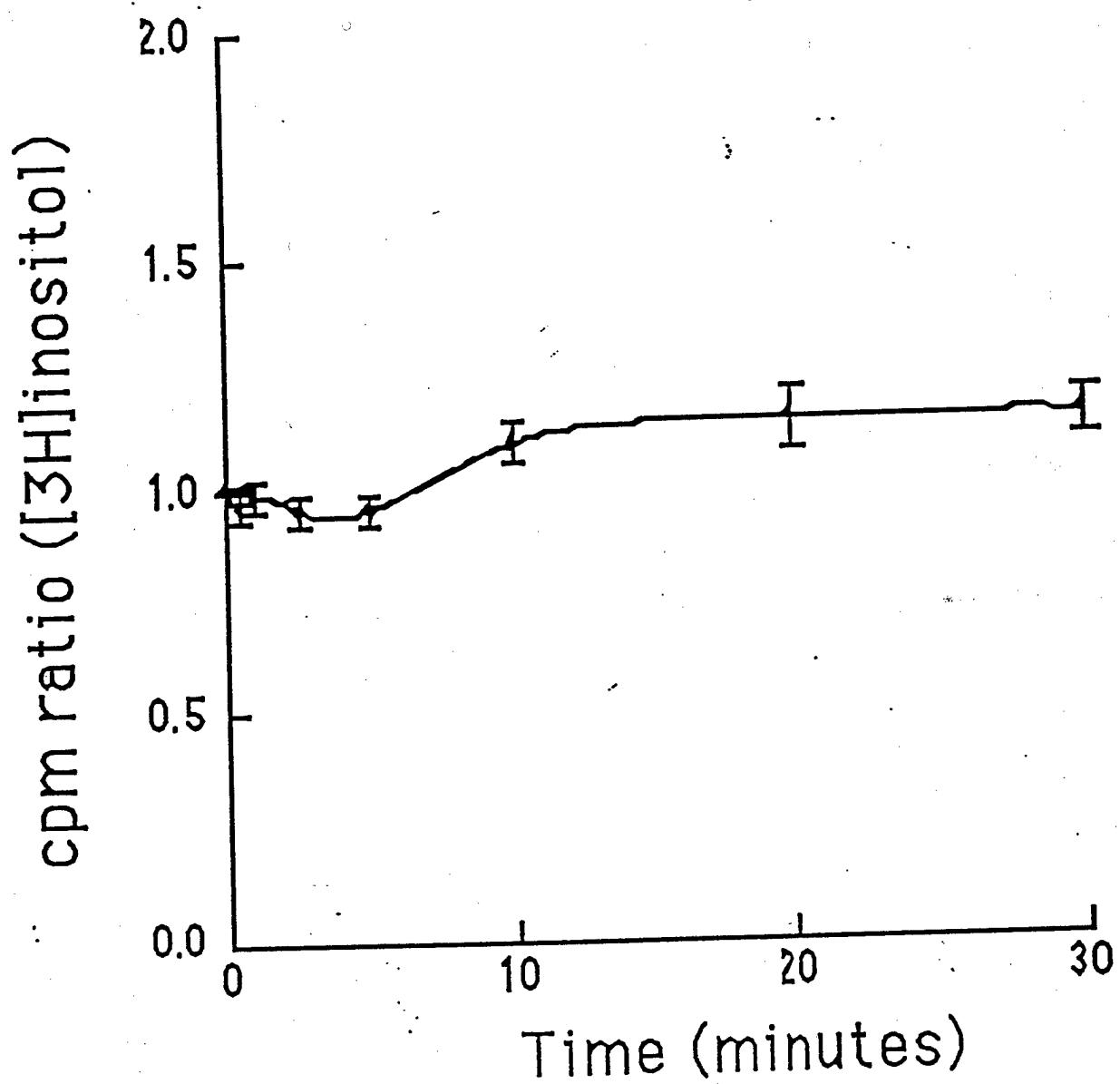
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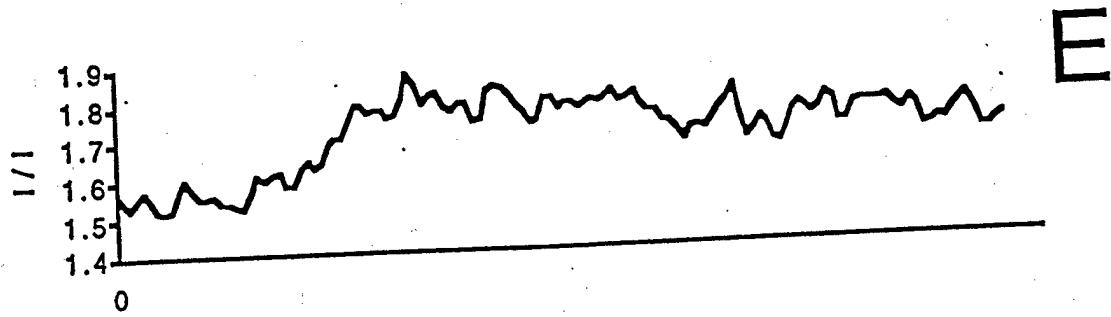
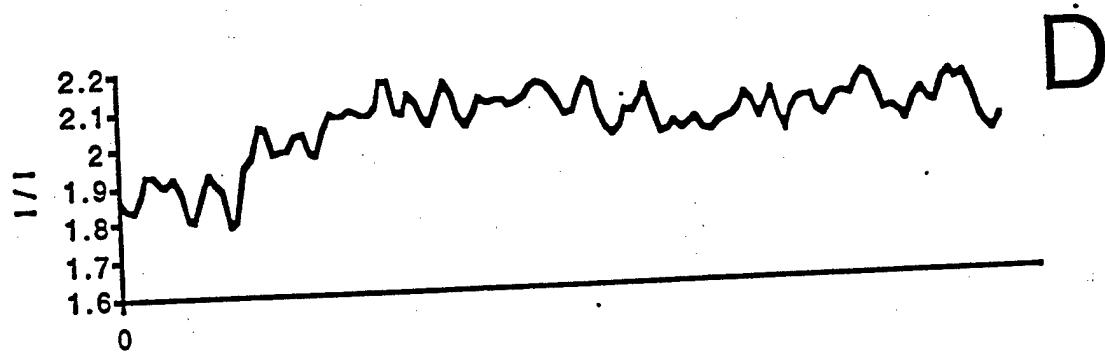
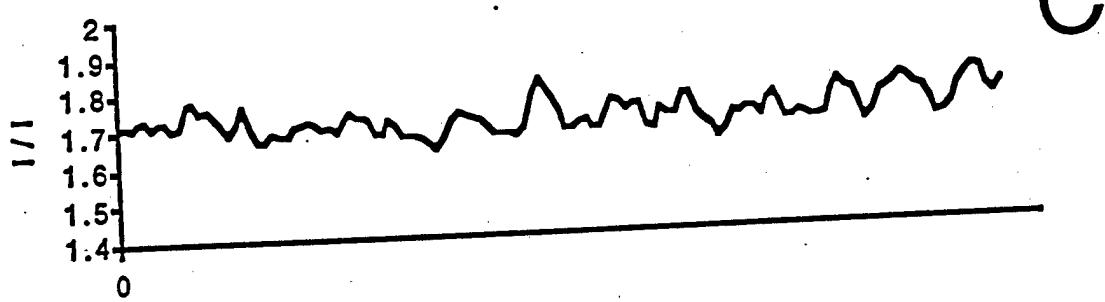
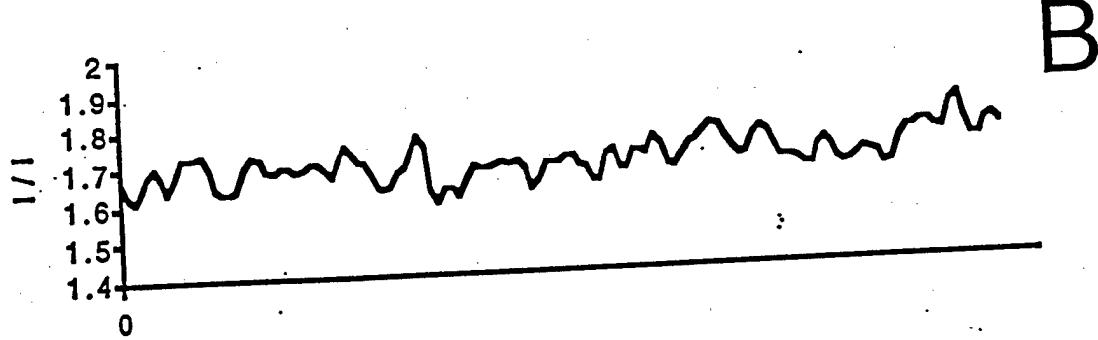
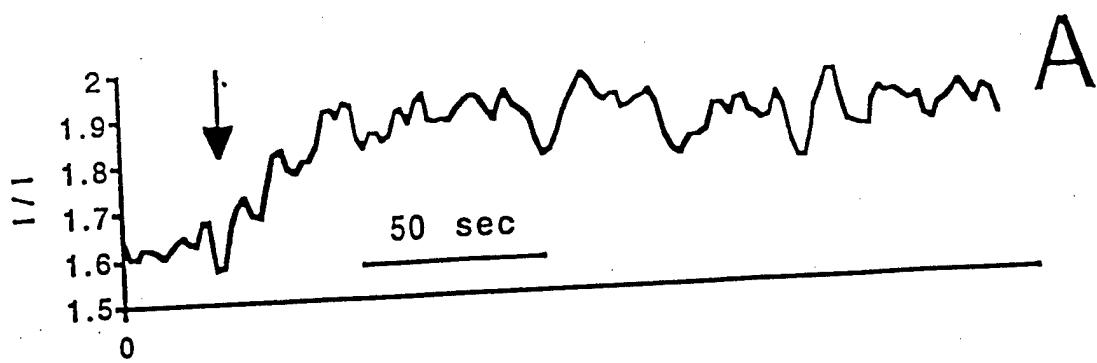




1 ATGGCGACGCTAACTCTTAAACATTAAGGTTCTCTGAGACAAATGCCATTTATCCGGCCGATGTTGAGCTTTACGCCACGAT
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3 CTCCTACTAACACGAGCTTGCATCTTCTGGGCTGCTGATCAGCTGCTCTTGGGATATAAGGCCACCAAAATCATCAGCTTTAAAGA
4 S V T K Q O W F D L F S D V H A V C L W D D K G P P K I H Q A L K E
5 CAGATATTCTTACGTTTAAACAGGCAAGGGACGGAACTGAGCATCAGATGATAAGCTTGCATAAAACGATAATCTGCAATGCCAAAGTC
6 P D I L E F I K Q A Q A R V L S H Q D D T A L L K A Y I V E W R K E
7 TITACACAAATGTGATTTAACCAAACTTTCTGCACTAGAGATTCTTAATGGTAAACAGGGCAGCAATAAAATCATGTTGAAAGACAGTA
8 P F I Q C D I L P K ? F C Q L E I T L H G K Q G S N K K S H V E D S
9 TCTTCGAAGCTTATGCTGAAACATGGAAAGTACTGATCTTTGCAACATAAAACAGACTCCACAGATACTGCAATGAACTGGTCATGCTGAAAG
10 P I V R K L M I D F W N E S I F S N ? K N R ? P D S A N K L V H A E R
11 ATGGGAGACGCTTCTGATCTCAACTGGTTATGGGATAAAATCTTAATGTTACCTTCTGATCTCTGAGGATACTCCAAATTATAGGGC
12 P L G E A F D S Q L V I G I K K S Y V Y L C S N P E D K L P I Y R D
13 ATTGGGAAAGGGATACCTGGATCCCCGAAAGATTTATGAAACACAGGGCCCTGTTTACAACAAAGCTTCCGAAATTGAAATATAGGGC
14 P N F E K A Y L D S P E R F Y R T Q A P S F L Q Q N G V P N Y L K Y
15 CAGATGCTCCATTCCAAAGAAGAAAACGGACACTACGTTTACAGAAAGACGAGATGTTACTCCGTTAACACTCATGGATGCTGTTAA
16 P D A R F F E E E K R A L R F L E T R R E C Y S V E A L H E C C V N
17 JGCCCTGGTGATCATTTAACGAAACTATCTAGCTGATGCCAGGGCTGATCAAAAGTGAACACTGAAACTGAAACTTAACTTAACTGTTCAATTGATG
18 P A L V T S F K E T I L A E C P G V I K K N E T E K I P L M F S L M
19 GACAAAGTTCTAAATGTTAAACGCAATGTTGAACTGAGGAAACATATCATTAGCTGTCGGCTGGCAGATAAGTGCAGCTGCTGAAACTATTA
20 P D K V P N C I K P M L K N L E E H I I S A G L A D H V A A A E T I
21 CTACTGACTCTGAGAAATGGTGGAGCTTACACTTAACTTAACTGAAACTCTGCTGAAAGAAACGTTTCTGAGATGATCCACGATTCTAC
22 P T T D S E K Y V E Q L I T L F N R F S K L V K E A F Q D D P R F L I
23 TCGAAGAGATAACGCTATAAAGCAGTTGTTAATGATGTTACCATTTAACTGAAATTGAACTTGGAAAGGGGGTGGGATAAAACTCAGGCT
24 P A R D K A Y K A V V N D A T I F K ? E L P L K Q K G V G L K T Q P
25 GATCAGAAATGCGCTGATCATTTAACGAAACTATCTAGCTGATGCCAGGGCTGATCAAAAGTGAACACTGAAACTGAAACTTAACTGATGCTGAA
26 P D K V P N C I K P M L K N L E E H I I S A G L A D H V A A A E T I
27 TTAACGAGCTGCTCTGACTTAATTATGATGTTACCATTTAACTGAAATTGAACTTGGAAAGGGGGTGGGATAAAACTCAGGCT
28 P A R D K A Y K A V V N D A T I F K ? E L P L K Q K G V G L K T Q P
29 GCGGCAACTGAAATGAAACATGGTGGAGCTGATGAAAGTGGTATGCCAGGGATTATGAAACAGCTTCTGAGATGTTGAGGACATA
30 P A D S E I E X N M V E W L K V G M ? A D Y V N K L A R M F Q D I
31 AAGGATCTGAGGATTTAACGCTTGGAAAGGAACTGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAA
32 P K V S E D L N G A F K E X H K N N X L A L P A D S V N I K I L N A
33 GCGCTGGCTCAAGAAGTTCTGAGAAAGCTTCTGAGGAACTGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAA
34 P A W S R S S E K V F V S I E T E D L I P E V E K F Y K K N H S
35 TGGGAAATTTACATGGATCATCTGATGAAAGGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAA
36 P G R K L H H H L M S N G I I T F K N E V G Q Y D L E V T T F Q L
37 GCTGATCTGCTGATGAAACGAAACCCAGAGGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAA
38 P A V L F A H N G R ? R E K I S F E N L K L A T E ? P D A E L R R T
39 TATGGCTTAACTTCCAAACGCAACGGGAACTTGTGAACTGAACTGGGAACTGAACTGGGAACTGAACTGGGAACTGAACTGGGAACTGAA
40 P G L W S L V T F P K L K R Q V L L Y E P Q V N S P K D F T E G T L F S
41 ACTGAAACAGGAATTCAGTTAAACGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAA
42 P V K Q E F S L I K N A K V Q K K G K I N L I G R ? L T T E R M R
43 GAGAAAGAAATGAACTGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAA
44 P E E N E G I V Q L R I L R T Q E A I I I Q I H K M R K K I S N A Q
45 TCGAGACTGAAATTAGTGAATTTAACGATGTTCTGCAACAAAAGGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAA
46 P Q ? E L V E I L K N M F L P Q K K M I K E Q I E W L I E H K Y I R
47 AGAGATGAAATGCAATCATCACATTACATGAAATATGAAATATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAA
48 P R D E S D I K T F I Y H A
49 TCTGAAAGTTGCTGAGGAGAAAGGTTATTCGACTTCTGATCATATAATTATATCTGCTCTTACCTGACAAJACACACCTGAAATTGAGGAT
50 P C C T A G T A C C G G G C C G A G T G C T G C A A T T A T G C T T C T G C G A A G G C A G G T G T G S S G T G A S T T C T C C C T C G A A A G C G G G







**BREAST CANCER CELLS EXPRESS NORMAL FORMS OF ALL
VASOPRESSIN RECEPTOR SUBTYPES PLUS AN ABNORMAL V2
RECEPTOR.**

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Abstract

Vasopressin is reported to influence the growth of breast cancer cells, and we and others have provided evidence that an autocrine loop involving vasopressin is present in perhaps all breast cancers. The current study was undertaken to examine possible vasopressin receptor subtypes taking part in this autocrine loop as it exists in MCF-7 cells in culture. RT-PCR demonstrated that mRNAs for all currently recognized vasopressin receptor subtypes (V_{1a} , V_{1b} , and V_2) are expressed by these cells. Cloning, and DNA sequencing over the entire open reading frame (ORF) of each mRNA revealed that normal sequences representing each receptor were present. However, in addition to these normal structures, an abnormal mRNA for the V_2 receptor was also expressed. This contains a sequence corresponding to intron 2 of the gene and is apparently the product of incomplete splicing. Such an mRNA, also found by us to occur in small-cell lung cancer cells, would be expected to give rise to a truncated and C-terminally altered "diabetic" form of receptor protein. Western analysis revealed that all three normal mRNAs gave rise to proteins of sizes compatible with them being functional receptors. The abnormal V_2 receptor mRNA also gave rise to proteins, which are presumed to be non-functional. The presence of all three normal vasopressin receptors in these breast cancer cells that also produce vasopressin suggests the autocrine loop of the peptide in breast tumors is multi-functional in nature. Vasopressin might then be expected to exercise its mitogenic influences through both vasopressin V_{1a} and V_{1b} receptors, producing changes in phospholipases C, D, and A_2 , in protein kinase C, and in Ca^{++} mobilization. However, it may additionally exercise a growth-inhibitory role through

normal V_2 receptors producing rises in cAMP and activation of protein kinase A.

INTRODUCTION

Vasopressin is reported to have growth-promoting activity on cells of the breast cancer cell line MCF-7 (1). Such an action would require these cells to express at least one subtype of vasopressin receptor, presumably the V_{1a} receptor subtype, but this has not yet been demonstrated.

Additionally, no attempt has been made to discover if two other receptor subtypes, vasopressin V_{1b} and V₂ receptors, are also present. While all three receptors are G-linked proteins possessing seven membrane-spanning domains, vasopressin binding to V_{1a} or V_{1b} subtypes promotes phospholipase activities while binding to the V₂ subtype primarily promotes adenylate cyclase activity and the formation of cAMP. Recently we were able to show that both classical and variant forms of small-cell lung cancer (SCLC) express all three receptor subtypes, plus a putative receptor hVACM (2,3). However, while a normal V₂ receptor subtype was apparently manufactured only by classical SCCL, both classical and variant SCCL generated an abnormal form of this receptor, apparently the product of incomplete posttranscriptional processing. In the current study, RT-PCR, cloning, and DNA sequencing were employed to obtain mRNA sequences that encompassed the entire open reading frame (ORF) of each of the human vasopressin V_{1a}, V_{1b}, and V₂ receptor subtypes. Additionally, antibodies specific to each receptor subtype were employed in Western analysis to examine the nature of protein products resulting from mRNA translation.

RESULTS

RT-PCR, Cloning and DNA Sequencing

Using forward and reverse primers selected to provide overlapping sequences covering the entire open reading frames (ORFs) of vasopressin V_{1a}, and V_{1b} mRNAs, RT-PCR of total RNA preparations from MCF-7 cells yielded, in each case, a single product of the expected size predicted from the cDNA for the human forms of these receptors derived from liver (4) or blood vessels (5), from pituitary (6,7); and from small-cell lung cancer (3). All of the products were generated from RNA and not DNA template because no intronic segments that these products spanned were evident. The three products obtained for the V_{1a} receptor (454, 669, and 858 bp), and the four obtained for the V_{1b} receptor (903, 768, 535, and 535 bp) are shown in Figure 1. However, RT-PCR with forward and reverse primers selected to obtain overlapping sequences covering the entire sequence of vasopressin V₂ mRNA (Figure 1) gave not only normal products (8), but also one abnormally sized product. The abnormal form was obtained as a second product when primers spanning intron 2 were used and was larger by the size of this intron (~100 bp) than the size of 862 bp, predicted from the structure of V₂ receptor mRNA. A similar product was earlier reported by us to be a product of small-cell lung cancer (3). The RT-PCR products representing V_{1a}, V_{1b}, and V₂ receptors of MCF-7 are represented in Figure 2. Cloning and sequencing of all V_{1a} and V_{1b} related products, and V₂ related products of predicted size, showed them to collectively provide a complete characterization of human V_{1a} mRNA for the MCF-7 breast cancer cell line from -23 at the 5' end (23 bases prior to the reading frame)

through 1224 at the 3' end (18 bases beyond the reading frame), for V_{1b} mRNA from 123 bases beyond the 5' end to 52 bases beyond the 3' end, and for V_2 mRNA from 32 bases beyond the 5' end to 53 bases beyond the 3' end. The sequences for the vasopressin V_1 receptor mRNAs had exact sequence homology with the sequence of human V_{1a} mRNA and the sequence of human V_{1b} mRNA published earlier by us and others (3,4,6,7). One sequence of human V_2 receptor mRNA from breast cancer cells was identical to that published by us and others for normal human tissues (8,9). Additionally, an enlarged product of the V_2 receptor was found to contain the entire 106 bases of intron 2 in addition to sequence for V_2 mRNA.

Western Analysis.

Western analysis using of the protein A-isolated IgG_{2b} preparation of polyclonal antibodies against the V_{1a} receptor (Vivian 3) revealed the presence of major protein bands at approximately 87 KDa, 82 KDa, 57 KDa, and 45 KDa, and 30 KDa, while use of isolated polyclonal antibodies to V_{1b} receptor (Bivily 3) gave a prominent protein bands at approximately 82 KDa, 78 KDa, 42 KDa, and 35 KDa. Western Analysis with polyclonal antibodies to the vasopressin V_2 receptor (Rocket 3) demonstrated prominent protein bands at approximately 47 KDa, 41 KDa, and 30 KDa. These could represent products of both the normal V_2 mRNA and the abnormal V_2 mRNA because both types of receptor proteins (and some of their metabolites) should react with Rocket antibodies that recognizes an epitope in the N-terminal region. When antibodies against the abnormal vasopressin V_2 structure (Abner 3) were used on MCF-7 cell extracts,

prominent protein bands at 81 KDa, 48 KDa, 43 KDa, 40 KDa, and 34 KDa, were displayed. These immunoreactive proteins would represent only the abnormal receptor plus glycosylated and N-terminally truncated metabolites because Abner antibodies specifically recognize the abnormal C-terminus. Data from Western analyses performed using the ECL procedure and antibodies against V_{1a}, V_{1b} and V₂ receptor proteins from MCF-7 breast cancer cells are illustrated in Figure 3.

DISCUSSION

Breast cancer, exemplified here by MCF-7 cells, is shown for the first time to express gene products for not one, but all three of the currently recognized receptor subtypes (V_{1a} , V_{1b} , and V_2) for the hormone vasopressin. Additionally, an mRNA for the putative human vasopressin receptor known as HVACM (10) was also shown to be expressed by MCF-7 cells, but details of these studies will be presented elsewhere. With respect to V_{1a} and V_{1b} receptor subtypes, a single mRNA is produced and each was found to have sequence identity with those expressed by normal tissues (4,6). For the V_2 receptor subtype, *both* a sequentially normal form of mRNA (8,9), and a sequentially abnormal formal of mRNA were present in these cells.

The abnormal mRNA expressed by MCF-7 cells is identical to the larger form of V_2 receptor mRNA produced by small-cell carcinoma (3). This mRNA contains the entire structure for intron 2 of the gene, and is apparently the product of incomplete splicing (8). The introduction of a *tag* stop codon into the reading frame, a short distance from the 5' end of the transcribed intron commencing at base position 1359 of the gene, leads us to expect this mRNA will translate into a C-terminally truncated receptor lacking the seventh transmembrane domain. That this mRNA is translated into protein products is supported by the presence in MCF-7 cell extracts of proteins immunoreactive with antibodies solely recognizing this abnormal truncated C-terminal structure. A similar receptor, lacking the seventh transmembrane domain and C-terminus of the normal V_2 receptor, is predicted to be produced through a single base substitution in the

inherited form of nephrogenic diabetes insipidus known as the "Utah" type (11). Since the Utah receptor appears to be non-functional, it is likely that the abnormal tumor receptor is either also non-functional, or serves as a "null" receptor if able to bind peptide and be expressed at the surface of cells. However, the studies of Sadeghi et al. (12) suggest that receptor proteins lacking the seventh transmembrane domain might not become components of the plasma membrane.

Both normal and abnormal mRNAs of MCF-7 breast cancer cells were shown to be translated into receptor proteins that are recognized through interactions with specific antibodies. The protein products of all three normal mRNAs seem to be of sizes (30-50 KDa and 70-87 KDa) compatible with them being functional receptors (4,7,8,13). The expression by breast cancer cells of both V_1 and V_2 receptors implies that vasopressin can initiate a whole range of intracellular cascades that include activation of phospholipases and protein kinase C, as well as activation of adenylate cyclase and protein kinase A (14-19).

A common feature of breast cancer would seem to be an autocrine loop involving vasopressin, because coupled with the current findings we have earlier obtained evidence that vasopressin is produced by apparently all breast cancers (20). The growth promoting actions reported for vasopressin, on MCF-7 breast cancer cells by Taylor and coworkers (1), and on mammary tumors by Chooi and coworkers (21), are presumably exercised through V_{1a} and/or V_{1b} receptors. These receptors are reported to activate phospholipases A₂, C, D, and protein kinase C, raise intracellular free-Ca²⁺, and increase phosphorylation of

MAP kinase and of FAK kinase, in normal tissues and in small-cell cancer cells (). All of these transduction cascades have been associated with cellular mitogenesis (14-18). However, Taylor and coworkers (1) also showed that vasopressin, can inhibit the growth of MCF-7 cells. We believe there is a growing body of supportive evidence that this negative influence on growth of the peptide could be exercised through normal vasopressin V₂ receptors, and have earlier proposed this for small-cell lung cancer (3).

In support of a vasopressin V₂ receptor-related mechanism being involved with the growth inhibition of breast cancer cells are the earlier findings that growth inhibition, as well as growth promotion and cellular differentiation in tumors can be associated with changes in cAMP (22,23,24). More recently and significantly, Cassoni et al. (25) have demonstrated that inhibition of a breast cancer cell line is produced by rises in cAMP. Since vasopressin raises cAMP by activating V₂ receptors, it seems likely the peptide can inhibit growth through this mechanism. If this is indeed the case, it means vasopressin through its different receptors is exercising a multifaceted, rather than a simple mitogenic role in the growth and survival of breast cancer, and can best be described as an autocrine growth *modulatory* agent, in much the same way it is regarded as a *neuromodulatory* agent in the central nervous system. It is also possible such eclectic properties are exhibited not only by vasopressin, but by most, or all, other autocrine tumor growth factors.

[The existence of a range of actions on breast tumors by vasopressin would open the way for the development of a range of new therapeutic approaches for treating this disease, that include use of peptide and non-peptide analogs of DDAVP to arrest tumor growth.]

MATERIALS AND METHODS

Cell Culture and Human Tissues

The breast cancer cell line MCF-7 was purchased from the American Type Tissue Culture Collection (Rockville, MD). This cell line has been shown to express vasopressin gene-related products by immunocytochemistry (26), and by Western analysis (unpublished data). Cells were maintained in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO.) supplemented with 10% FBS (Hyclone, Logan, UT) at cell densities of 10^5 - 10^6 cells/ml in a humidified atmosphere of 5% CO₂ at 37°C.

RNA Isolation and RT-PCR

Total RNA was isolated from cells using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). This total RNA (1-5 μ g) was added into a SuperScript preamplification system (Life Technologies, Inc.) for the synthesis of the first cDNA strand by using an oligo (dT) primer and reverse transcriptase (400 U), and the product used directly for PCR. PCR was performed in a thermocycler (ERICOMP, San Diego, CA). The reaction mixtures were overlaid with 50 μ l of mineral oil and subjected to an initial denaturation at 96°C for 2 min., followed by 30 cycles comprising a denaturation step at 96°C for 30 sec.; a step for annealing the primers to the template at 58°C for 1 min. 30 sec.; and an extension step at 72°C for 1 min. 30 sec. At the completion of the cycling reaction, an additional extension step at 72°C for 10 min. was performed. The primers selected for PCR and sequencing of V_{1a}, V_{1b} and V₂ receptors are summarized in

Figures 1a, 1b, and 1c. These primers were synthetic 18, 19, 20, and 22 oligomers designed to yield overlapping PCR products of 454, 669, and 858 base pairs (bp) for V_{1a} receptors; synthetic 18 and 20 oligomers yielding overlapping PCR products of 903, 768, 535, and 589 bp for V_{1b} receptors; and synthetic 20 oligomers providing overlapping PCR products of 512 and 862 bp for V₂ receptors (). These PCR products for V₁ and V₂ receptors collectively spanned the entire open reading frame of the receptor mRNAs. PCR products were extracted with an equal volume of chloroform and examined on 2% agarose gels.

Cloning and Sequencing

PCR products (1 μ l, 4-12 ng) of vasopressin receptors were ligated into a pCRTM vector and 2 μ l of the ligation mixture transformed in One ShotTM Competent Cells using a TA Cloning Kit (Invitrogen, San Diego, CA). Plasmid clones were prepared with a WizardTM Minipreps DNA purification system (Promega, Madison, WI.), and screened by EcoR I digestion and agarose gel electrophoresis. At least two positive clones of each PCR product were chosen for double strand cDNA sequencing with a DyeDeoxyTM Terminator Cycle Sequencing Kit (Applied Biosystems, Perkin Elmer). The primers chosen for PCR amplifications as described in Figure 1, and vector universal primers (M13 Forward and M13 Reverse), together served as sequencing primers. The protocol for sequencing cloned cDNA (1 μ g) was as follows: 96°C, 2 min.; 25 cycles at 96°C, 15 sec., 50°C, 15 sec., and 60°C, 4 min. The products were purified with Centri-Sep spin columns (Princeton Separations, Adelphia, NJ). Automated DNA sequencing was performed using a Model 373 DNA Sequencer from Applied Biosystems.

Polyclonal Antibodies and Western Analysis

Rabbit polyclonal antibodies to the human vasopressin V_{1a}, V_{1b}, and V₂ receptors, and an abnormal form of V₂ receptors, were generated using procedures similar to those described previously(2,3). Decapeptide amides, nonapeptide amides, or decapeptides representing 8-10 amino-acid unique sequences in each of the four receptor forms with an added N-terminal tyrosine (to permit iodide labeling), when this amino-acid was not already present in the sequence chosen, were used as antigens (2,3). Antibodies from antisera (Vivian for V_{1a} receptor, Bivily for V_{1b} receptor, Rocket for V₂ receptor, and Abner for the abnormal V₂ receptor) obtained in this manner were isolated as IgG2b fractions from protein A-Sepharose (Sigma) chromatography for employment in Western blot analysis at concentrations of 10 µg/ml. Samples for Western analysis were prepared by sonicating cells (0.4 - 1.2 x 10⁷) in 1.0 ml of Lysis Buffer (10 mM Tris HCl, 5 mM EDTA, 50 mM NaCl, 30 mM Na PO₄, 50 mM Na F, 0.1 mM Na₃VO₄, 1 mM PMSF, 0.2 I.U./ml Aprotinin, 0.1% Triton X-100, pH 7.6). Homogenates were centrifuged for 2 minutes at 12,000xg and ambient temperature. Soluble extracts were mixed 1:1 with 2x SDS/ Tris-HCl sample buffer (pH 8.7) containing 50 mM DTT, and proteins reduced by heating at 100°C for 5 min. These proteins were then subjected to SDS-PAGE electrophoresis on 12% gels at pH 9.3 using the method of Laemmli (29). Separated proteins were electrophoretically transferred in 20 mM Tris glycine to Immobilon PVDF membranes. These membrane transfers were dried and incubated for 16 h at 4°C, with rabbit polyclonal antibody preparation against V_{1a}, V_{1b} or V₂, or V₂ abnormal, receptors (see above) in pH 7.4 Tris HCl buffer (10 mM Tris HCl, 100 mM NaCl, 5% BSA, and 0.1%

Tween 20). Following washings (x3) in the pH 7.4 buffer, membrane transfers were incubated with a goat anti-rabbit IgG-horseradish peroxidase conjugate for 1 h. Each was removed by washing (x3) in Tris HCl buffer, pH 7.4. Transfers were sometimes placed in a 1:50 dilution of 33% H₂O₂ in methanol for 30 min. before blocking (to destroy endogenous peroxidase activity in the sample). Immunoreactive proteins in peroxidase-treated transfers were visualized using an ECL Western Blotting Detection System (Amersham Life Sciences, Arlington Heights, IL) with exposure of X-ray film from 30 sec. to 120 sec. Prestained SDS-PAGE standard proteins (Sigma) were employed as molecular size markers.

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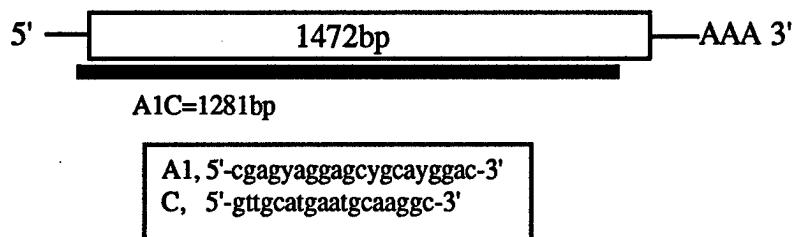
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Figure 1. Synthetic primers selected for RT-PCR and sequencing from the MCF-7 breast cancer cell line (a). the vasopressin V1a receptor; (b). the vasopressin V1b receptor and; (c). the vasopressin V2 receptor. These oligomers generated overlapping PCR products that collectively spanned beyond the entire reading frame of the receptor mRNAs and introns of the genes. Sequences obtained from these products provided sequences identical to sequences of mRNAs published earlier for normal human tissues by us and others (), and, in addition, a partially spliced V₂ mRNA that retains intron 2 of the gene and can give rise to a C-terminally truncated receptor. All of these forms were previously shown by us to be expressed by small-cell lung cancer ()�.

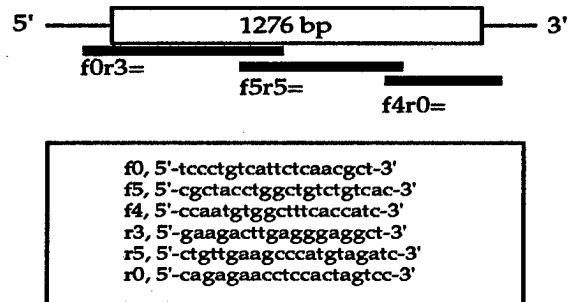
Figure 2. RT-PCR products obtained with a total RNA preparation from MCF-7 breast cancer cells and separated on 2% agarose. Selected primers for V1a, V1b and V2 receptors (see Figure 1) yielded overlapping cDNAs that provided the sequence for the entire reading frame of human vasopressin V1a, V1b, and V2 receptor mRNAs. (a) V1a receptor: lane 1, 100 bp DNA ladder; lane 2, 3 and 4, 454 bp, 669, and 858 bp of V1a products of expected size; (b) V1b receptor: lane 1, 100 bp DNA ladder; lane 2, 589 bp product of expected size obtained with primers f2 and r0; lane 3, 535 bp product of expected size obtained with primers f2 and r2. (b) V₂ receptor: lane 1, 512 bp product of expected size obtained using primers A and B; lane 2 968 bp product of enlarged size obtained using primers C and D; lane 3, 862 bp product of normal lung obtained using primers C and D.

Figure 3. Western blot analysis from SDS-PAGE (12%) with an ECL detection system (Amersham) showing dithioerythritol-reduced proteins from MCF-7 cells that were immunoreactive with rabbit polyclonal antibodies: (a) Proteins of approximately KDa immunoreactive with antibodies against human V_2 receptor (Viavian 3); (b) Proteins of approximately 39 KDa immunoreactive with antibodies against human V_{1b} receptor (Bivily 3); (c) Proteins of approximately 12 KDa, 17 KDa, and 33 KDa immunoreactive with antibodies against human V_2 receptor (Rockie 3) and; (d) Proteins of approximately 36 KDa, 43 KDa, 50 KDa and 61 KDa immunoreactive with antibodies against an abnormal human V_2 receptor (Abner 3) . The transfers were first reacted with peroxide in ethanol to block endogenous peroxidase activity, and the PVDF membrane then blocked with a 5% BSA solution in Tris HCl-Triton. Incubation with the Protein A-isolated primary antibodies was performed for 16 h at 4°C, and incubation with the goat-antirabbit IgG-horseradish peroxidase conjugate (GIBCO/BRL) in Tris HCl-Triton was performed for 1 h at ambient temperature. Immunoreactive proteins were visualized on X-ray film exposed to the transfer for 2 min, 30 sec, and 1 min, respectively.

RT-PCR primers and products of V1a receptor in MCF-7 breast cancer cells



RT-PCR primers and products of V1b receptor in MCF-7 breast cancer cells



RT-PCR primers and products of V2 receptor in MCF-7 breast cancer cells

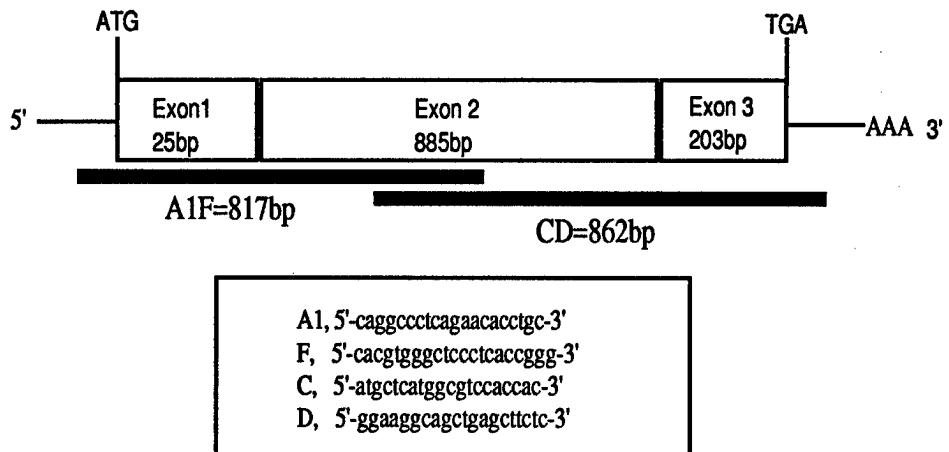


Figure 1

Figure 2 Not Yet Available

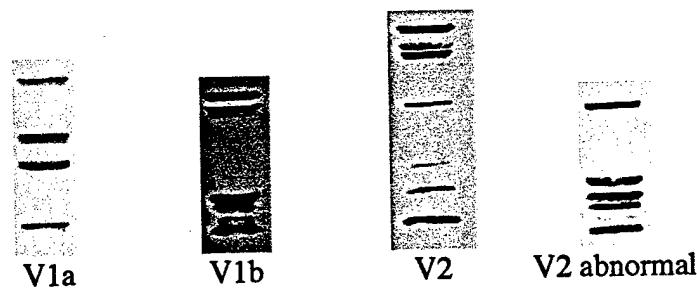


Figure 3

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EXPRESSION OF THE VASOPRESSIN GENE BY HUMAN BREAST CANCER. ((M.J. Fay, X. Yu, V. Memoli*, and W.G. North)) Departments of Physiology and Pathology*, Dartmouth Medical School, Lebanon, NH 03756.

The purpose of this study was to determine if the vasopressin gene is normally expressed by breast cancer, and if this expression leads to the formation of a cell surface marker for this disease. We have previously established in small-cell lung cancer that expression of the vasopressin gene leads to the formation of 20 and 42 KDa cell surface markers (NRSA, neurophysin-related cell surface antigen). Vasopressin gene expression by 19 breast tumors, prepared as acetone fixed specimens, was examined by avidin-biotin (ABC) immunohistochemistry using antibodies directed against vasopressin (VP), the bridging peptide region of the vasopressin precursor (pro-VP), vasopressin-associated human neurophysin (VP-HNP) and vasopressin-associated glycopeptide (VAG). All tumors tested stained positively with anti-VP, and anti-VAG. Of 14 tumors examined with anti-(pro-VP), 11 demonstrated positive staining, while only 1 of 19 tumors was positive for anti-(VP-HNP). Surrounding normal breast tissue gave negative staining with all of these antibodies. Western-blot analysis from SDS-PAGE using 4 biopsied human breast tumors and a monoclonal antibody directed against VP-HNP revealed protein products with apparent molecular weights of 20 and 40 KDa. Indirect immunofluorescence with anti-VAG and flow cytometry analysis using live MCF 7 breast carcinoma cells revealed cell surface immunoreactivity which was 7 fold greater than negative controls. These studies suggest that vasopressin-gene related products are commonly expressed by breast carcinoma cells, and that this expression in cultured cells leads to the production of a cell-surface antigen.

3

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5

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9

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Vasopressin and Breast Cancer: Gene expression and Trafficking.
William G. North, Michael J. Fay, and Jinlin Du, Dartmouth Medical School,
Lebanon, N.H. 03756 USA.

We earlier discovered that the vasopressin gene expression occurs in probably all breast cancers, that this expression apparently arises as part of the carcinogenesis process in the mammary gland, and that 40 KDa and 20 KDa vasopressin-related proteins are generated as components of the plasma membrane in breast tumor cells. We have named the membrane proteins GRSA (glycopeptide-related cell surface antigens). We have now examined aspects of vasopressin gene expression and the processing of gene-associated products in MCF-7 and ZR-75, using RT-PCR, cloning, DNA sequencing, sucrose-gradient fractionation, Western analysis, and flow cytometry. Results obtained have led us to the following conclusions:

- GRSA surface markers originate through the expression of both normal and abnormal vasopressin genes. This is because RT-PCR products of normal and increased size, as well as with normal and abnormal sequences, were obtained;
- trafficking of GRSA proteins to the cell surface is controlled by factors additional to structural elements within these proteins. This is because both abnormal 40 KDa proteins as well as seemingly normal 20 KDa provasopressin are packaged into neurosecretory vesicles;
- abnormal posttranslational processing of vasopressin-related proteins by tumor cells is not due to their inability to express intravesicular processing enzymes. This is because we were able to demonstrate that functional forms of prohormone convertase 2 (PC2) and carboxypeptidase E (CPE) are probably produced by these cells;
- GRSA proteins contain vasopressin and neurophysin structures, as well as the glycopeptide moiety of provasopressin. This is because antibodies to vasopressin, human vasopressin-associated neurophysin (VP-HNP) and vasopressin-associated glycopeptide (VAG), all react with both 40 KDa and 20 KDa protein forms, and;
- GRSA proteins can be potentially used in new immunotherapeutic treatments of breast cancer. This is because the proteins, as components of viable cells in vitro, react with specific antibodies.

63.4

PRODUCTION AND PROCESSING OF VASOPRESSIN GENE-RELATED PROTEINS BY NEUROENDOCRINE TUMORS. W.G. North & J. Du. Dept. of

Physiol., Dartmouth Med. Sch., Lebanon, NH 03756.

We have discovered that vasopressin (VP) gene-related proteins are most probably universal lineage markers for not only small-cell carcinoma of the lung (SCCL), but also breast cancer. Unlike their production by neurons, most (>90%) of these proteins are not packaged into secretory vesicles by these tumor cells, but instead are trafficked to the plasma membrane where they uniquely form surface antigens (NRSA). RT-PCR, cloning, sequencing, immunocytochemistry, Western analysis, and flow cytometry, have allowed us to reach the following conclusions about these tumor proteins:

- NRSA originates from of both normal and abnormal VP genes;
- VP gene expression is a likely feature of the carcinogenic process that generates tumors such as SCCL and breast cancer;
- errors take place in transcription that probably lead to tumor-specific abnormal posttranslational processing;
- NRSA arises through both normal and abnormal posttranscriptional processing;
- trafficking of NRSA to the cell surface is controlled by factors additional to structural elements within the proteins translated;
- abnormal processing of proteins by tumors is not due to their inability to express intravesicular processing enzymes;
- changes in tumor differentiation (or drug resistance) does not affect the nature nor the degree of expression of NRSA.
- membrane models for NRSA require VP, neurophysin, and glycopeptide elements to be extracellular.

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2378

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Abstract title Evidence for the expression of a novel vasopressin-activated calcium mobilizing receptor (HVACM) in human breast cancer and lung cancer cell lines

Authors including presenter (Please underline presenter's name)

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Dartmouth Medical School

2 William G. North, Ph.D.

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3 Jinlin Du, M.D.

Dartmouth Medical School

4 Michael J. Fay, Ph.D.

Dartmouth Medical School

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The purpose of this study was to determine if a human homologue of the rabbit vasopressin-activated calcium mobilizing (VACM-1) receptor is expressed in human cancer cells. Vasopressin (AVP) may be involved in human breast cancer and lung cancer pathophysiology, as an autocrine/paracrine factor. AVP can act through four classes of receptors: V₂, V_{1a}, V_{1b}, and the recently cloned VACM-1, a structurally unique member of this group that contains a single transmembrane domain. (Recently, a highly homologous cDNA, termed HVACM, was cloned from human placental mRNA.) AVP induced an increase in intracellular free calcium in the breast cancer cell lines MCF-7, T47-D, and ZR-75, and in the lung cancer cell line NCI H-146. Total RNA from these cell lines and normal human tissues (kidney and lung), was used for reverse transcription polymerase chain reaction (RT-PCR) and Northern blot analysis. RT-PCR, using two primer sets designed against the rabbit VACM-1 sequence, amplified bands of the predicted sizes of 674 bp and 193 bp in all cell lines and tissues tested. Direct sequencing of PCR products obtained from H-146 revealed a high degree of identity to the rabbit VACM-1 cDNA (90%) and the human HVACM cDNA (99.5%). Northern blot analysis revealed three distinct bands (3.5, 5 and 6.5 kilobases) in the cancer cell lines. In summary, we have demonstrated the presence of mRNA for a novel AVP receptor in human cancer cell lines and normal human tissues.

VASOPRESSIN GENE-RELATED PRODUCTS
IN THE MANAGEMENT OF BREAST CANCER.

William G. North, Ph.D., Michael J. Fay, Ph.D.,
Kenneth Longo, B.S., and Jinlin Du, M.D.

Dartmouth Medical School, Department of Physiology,
1 Medical Center Drive, 752E Borwell, Lebanon, NH 03756

There is currently no known universal marker system for breast cancer that can be utilized in tests for early detection, for tumor localization, and for targeted treatment. Most approaches in the management of this disease depend on mammography for detection, and combination chemotherapy and radiation for treatment. We discovered that all breast cancers we examined expressed the vasopressin gene, and set out to determine if this expression represented a universal marker system for the disease. We also have commenced examining the nature of the gene-related products generated by this expression, their role in tumor growth, and their potential usefulness in developing new methods for early detection and for rational treatments. Our approach has involved employing immunohistochemistry and a battery of our antibodies directed against different regions of the provasopressin molecule, methods of protein isolation and characterization, flow cytometry, reverse transcription followed by amplification through polymerase chain reaction (RT-PCR), DNA sequencing, sucrose-gradient fractionation, and radioimmunoassay. In our studies we have utilized surgical and biopsy specimens of breast cancer, normal breast tissue, breast fibrotic disease, and breast carcinoma *in situ*, and employed five breast cancer cell lines in culture.

Results obtained using immunohistochemistry have revealed that vasopressin gene-related products are very likely universal markers of early carcinogenesis in breast tissues. This is because all of 19 breast tumors examined gave diffuse positive immunostaining for different components of the provasopressin molecule, while no staining was obtained with normal breast tissues. No cases of polycystic disease examined, including typical and atypical hyperplasia, gave positive staining and this showed tumor immunoreactivity does not simply represent tissue proliferation. All cases of carcinoma *in situ* gave diffuse positive staining with antibodies against vasopressin-associated human glycopeptide (VAG) suggesting

Keywords: Vasopressin Gene and Carcinogenesis, Glycopeptide-Related Surface Antigen, Targeting, Vasopressin Receptors, Autocrine Growth Factor

This work was supported in part by the U.S. Army Medical Research and Material Command under DAMD 17-94-j-4288.

vasopressin gene expression is also common to this form of preinvasive breast cancer. Results obtained from protein analysis and sucrose-gradient fractionation studies, on breast cancer and the MCF-7 and ZR-75-1 cell lines, indicate vasopressin gene expression in breast cancer gives rise to unique major protein products of 40 KDa and 20 KDa that become components of the plasma membrane, and are largely (>90%) processed outside of secretory granules. We have named these proteins collectively GRSA (glycopeptide-related surface antigen) because for viable MCF-7 cells in culture they were found to react with our antibodies to VAG. Ongoing RT-PCR studies on MCF-7, T47D, ZR-75-1 cell lines, utilizing primers designed to produce cross-over products for the whole reading frame of vasopressin (VP) mRNA, have so-far allowed us to deduce that GRSA proteins are the products of at least two VP mRNAs, one of normal size and presumably generated from a normal gene, the other(s) containing an additional 600 bases upstream from Exon B and generated either from a normal gene through alternative splicing that includes a portion of intron 1 or from an abnormal gene with an insertion in Exon A. In our studies we have additionally found no evidence for cross-over between vasopressin and oxytocin genes in breast cancer. Although cellular trafficking of GRSA proteins is largely outside of secretory vesicles, we have determined breast cancer cells are capable of expressing proteolytic enzymes required in normal intravesicular processing. Primer pairs for amplification of cDNA fragments of prohormone convertases (PC) 1/3 and 2, and carboxypeptidase E (CPE) were used in RT-PCR performed on RNA from cell lines MCF-7 and ZR-75-1. For CPE primers, products of the predicted size were obtained from both cell lines, and DNA sequencing gave a sequence identical to that published for functional CPE of anterior pituitary. Similarly, a product of predicted size and normal structure could be amplified using PC 2 primers from MCF-7 cells, but not from ZR-75-1 cells. Neither cell line seemed to express mRNA for PC 1/3. While most VP gene expression culminates in GRSA protein production, some of it appears to produce vasopressin and VAG as secretory products. This is because, using our RIAs, we were able to show these products elevated in the plasma of 7 patients with breast cancer. RIAs for VP and VAG might therefore find a use in methods for detecting tumors and monitoring treatments. Vasopressin (VP) appears to be an autocrine growth factor for breast cancer. In this respect, we were have been able to demonstrate for T47D and ZR-75-1 cells, using Indo-1AM fluorescence and flow cytometry, that the peptide can increase intracellular free-Ca²⁺ in a dose-dependent manner. We were also able to show through Western analysis that VP can activate mitogen-activated protein (MAP) kinase in these cells. Although these effects both appear to be through a vasopressin (V₁) receptor mechanism, RT-PCR and DNA sequencing has been used by us to show that breast cancer cells are capable of expressing all four vasopressin receptor subtypes (V_{1a}, V_{1b}, V₂, and human VACM), as well as oxytocin receptors. BT 549, MCF-7, MDA-MB-231, T47D, and ZR-75-1 cells have featured in these receptor studies.

Our studies have therefore led us to the following conclusions: 1) the vasopressin gene is a universal marker of carcinogenesis in breast tissue; 2) vasopressin gene expression in breast cancer uniquely leads to the formation of surface GRSA proteins that are potential targets for immunotherapy; 3) breast tumors are neuroendocrine and most cause plasma elevations of vasopressin gene-related products that can be potentially used for detection and monitoring treatments; 4) vasopressin is an autocrine growth factor for breast cancer, and 5) expression of multiple VP receptors subtypes implies vasopressin plays a multifaceted role in tumor growth and survival. All of these conclusions speak to the future importance of vasopressin gene-related products for developing new and sensitive methods of detecting breast cancer and monitoring treatments, and new and successful immunotherapeutic interventions.

THE ROLE OF VASOPRESSIN AND OXYTOCIN HORMONES IN BREAST CANCER

Michael Fay, Ph.D., Jinlin Du, MD, Kenneth Longo,
and William North, Ph.D.

Department of Physiology, Dartmouth Medical School,
1 Medical Center Drive, 752E Borwell, Lebanon, NH 03756.

This laboratory has demonstrated that fixed breast cancer biopsy specimens exhibit positive immunoreactivity for vasopressin and oxytocin gene-related products using the technique of immunohistochemistry and antibodies directed against different regions of the vasopressin and oxytocin prohormones. In addition, both *in vitro* and *in vivo* research indicate that neuropeptides, like vasopressin and oxytocin, modulate breast cancer cell growth. Taken together these results suggest that vasopressin and oxytocin may serve as autocrine and/or paracrine growth modulators for breast cancer cells. However, the receptors and signal transduction pathways through which vasopressin and oxytocin act to influence breast cancer cell growth remain unknown. The purpose of this research is to determine if breast cancer cells express vasopressin and oxytocin receptors, and to evaluate vasopressin- and oxytocin-induced signal transduction in breast cancer cells.

To evaluate which vasopressin and oxytocin receptor subtypes are expressed by breast cancer cells the technique of reverse-transcription polymerase chain reaction (RT-PCR) was used with primer pairs specific for the oxytocin receptor, the V1a vasopressin receptor, the V1b vasopressin receptor, the V2 vasopressin receptor, and the vasopressin-activated calcium mobilizing receptor (VACM). The VACM and V1b receptor PCR products were confirmed by direct DNA sequencing. To study vasopressin and oxytocin induced changes in intracellular free calcium, breast cancer cells were loaded with indo-1 AM, and neuropeptide-induced changes in intracellular free calcium monitored over a four minute period using a Becton Dickinson Facsstar Plus flow cytometer [excitation 356 nm, emissions 405 nm (calcium bound indo), and 485 nm (free indo)]. To determine if vasopressin causes activation of the mitogen activated protein kinase cascade (MAP kinase), MCF-7 breast cancer cells were stimulated with vasopressin, and activated (phosphorylated) MAP Kinase evaluated by western blot analysis.

Keywords: Breast Cancer Cells, Vasopressin and Oxytocin, Vasopressin and Oxytocin Receptors, Signal Transduction.

This work was supported by the U.S. Army Medical Research and Material Command under DAMD 17-94-j-4131.

Using the technique of RT-PCR evidence was obtained for the expression of mRNA(s) for a number of vasopressin and oxytocin receptor subtypes in cultured breast cancer cell lines. Using two primer pairs based on the sequence of the VACM receptor, PCR products of the predicted sizes of 674 bp and 193 bp were amplified from MCF-7, T47D, and ZR-75 breast cancer cell lines. Using a primer pair based on the oxytocin receptor, a PCR product of the predicted size of 391 bp was amplified from BT549, MCF-7, MDA-MB-231, T47D, and ZR-75 breast cancer cell lines. From the ZR-75, BT549, and MCF-7 cell lines a PCR product of the predicted size of 862 bp was amplified using primers for the V2 vasopressin receptor. In addition, using the V2 receptor primers, a PCR product which is approximately 100 bp larger than expected was amplified from these three cell lines. It is believed that this PCR product represents an incompletely spliced mRNA species containing the second intron. Using Primer pairs that amplify a 239 bp PCR product for the V1b vasopressin receptor, a product of the predicted size was amplified from the MCF7 breast cancer cell line. Preliminary PCR results using a primer pair based on the V1a vasopressin receptor indicate that a PCR product of the predicted size of 408 bp was amplified from the T47D breast cancer cell line. The identity of the VACM and V1b PCR products has been verified by direct DNA sequencing of the PCR products. Northern blot analysis for VACM using RNA from the ZR-75, MCF-7, and T47D cell lines indicates RNA species of ~ 3.5, 5, and 6.5 Kb. Using indo-1 AM loaded ZR-75 and T47D breast cancer cells neuropeptide induced changes in intracellular free calcium was monitored using flow cytometric analysis. Vasopressin (0, 10 nM, 100 nM, and 1,000 nM) was administered after approximately 20 seconds of baseline. In both cell lines vasopressin at the 100 nM and 1,000 nM doses induced a rise in intracellular-free calcium as indicated by an increase in the 405nm/485nm ratio. At all the doses studied oxytocin (10 nM, 100 nM, 1,000 nM) did not cause a noticeable rise in intracellular-free calcium in the ZR-75 and T47D cell lines. Treatment of MCF-7 breast cancer cells with 100 nM and 1,000 nM vasopressin resulted in a dose-dependent increase in tyrosine phosphorylated MAP kinase as determined by Western blot analysis.

Both *in vivo* and *in vitro* results indicate that neuropeptides like vasopressin can serve as growth modulating agents for breast cancer. Research performed in this laboratory indicates that neuropeptides, like vasopressin and oxytocin, are produced by breast cancer cells. Collectively these results suggest that neuropeptide hormones may serve as autocrine/paracrine factors for breast cancer. The results obtained in these studies provide further support for a role of vasopressin and oxytocin as paracrine/autocrine factors for breast cancer since mRNA(s) for a number of receptors for these hormones are expressed in cultured breast cancer cells. Vasopressin treatment causes a rise in intracellular free calcium in two cultured breast cancer cell lines, suggesting that the hormone might be activating VACM, V1a, or V1b receptor subtypes. Experimental results obtained with the MCF-7 breast cancer cell line suggest that the influence of vasopressin on breast cancer cell growth observed *in vivo* and *in vitro* may be due to activation of the MAP kinase cascade. These results further support a role for neuropeptide hormones like vasopressin and oxytocin in breast cancer pathophysiology. Identifying hormones involved in breast cancer cell growth, the hormone receptors through which these peptides act, and the cellular changes associated with receptor activation is crucial to identifying novel strategies for the treatment of breast cancer.

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LOCUS XXXXX 1298 bp mRNA PRI 21-OCT-1997
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 and Clauzer,E.
 TITLE Molecular cloning, sequencing, and functional expression of a cDNA
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 JOURNAL J. Biol. Chem. 269, 3304-3310 (1994)
 REFERENCE 2 (bases 1 to 1298)
 AUTHORS North,W.G., Fay,M.J., Longo,K.A. and Du,J.
 TITLE Functional Vasopressin V1 Type Receptors are present in Variant as
 well as Classical forms of Small-Cell Carcinoma
 JOURNAL Peptides 18, 985-993 (1997)
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 AUTHORS Du,J., Fay,M.J., Longo,K.A. and North,W.G.
 TITLE Direct Submission
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 COMMENT Hirasawa, A. Biochemical and Biophysical Research Communications,
 203,72-79,1994

Thibonnier, M. Genomics, 31, 327-334, 1996.

FEATURES Location/Qualifiers

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 VRNEELAKLEJAVLAVTFAVALGNSSVLLALHRTPRKTSRMHLFIRHLSLADLAV
 AF
 FQVLPQMCWDITYRFRGPDWLCRVVKHLQVFGMFASAYMLVVMTADRYIAVCHP
 LKTL
 QQPARRSRLMIAAAWVLSFVLSTPQYFVFSMIEVNNVTKARDCWATFIQPWGSRA
 YVT"

WMTGGIFVAPVVLGTCYGFICYNIWCVNRGKTASRQSKGAEQAGVAFQKGFLA
 PCV
 SSVKSIISRAKIRTVKMTFVTVTA YTCWAPFFIQMWSVWDPMSVWTESENPTTTA
 LLGSLNSCCNPWYMFSGHLLQDCVQSFPCQNMKEKFNKEDTDSMSRRQTFYS
 NNR

SPTNSTGMWKDSPKSSKSIKFIPVST"

BASE COUNT 258 a 399 c 364 g 277 t

ORIGIN

1 cgagtagggag cggcaaggac agcaigcgic tcicccgcgg tcccgacgsg gggccctcgg
 61 gcaacccag cccaaagggg cctctggcca cggcgctgg caacacaaac cgggaggccg
 121 aagccctcgg ggggggcaac ggcccaccga gggacgtgcg caaccaaggag ctggccaaac
 181 tggagatcgc cggcgccgc gigacttgcg cggggccgt gcggggccac agcagcgac
 241 tgcggcctcgcacccggacg cccgcgaaga cggcccat gcacccttc aacccacacc
 301 tcaaggccggc cggccggcc gggcatttcc tccagggtgt gccgcgggg tgcgggaca
 361 tcaacctaccg cttccggggc cccgaciggc tgcggccgt gggtggccac tggcagggtgt
 421 tggcgttgtt tggcgtggcc tacaatgtgg tggcgtggcc agccggccgc tacaatcgccg
 481 tggccaccc ggcaaaatc tggcaacagc cccgcggccg tccggccic aigatcgccg
 541 ccggccgggt gcaatgcgc tggctggagca cggccggatc tccggccic tccatgtcg
 601 agggaaacaa tggccaaatc gcccggccat gcaatggccac ccacccatc cccgggggt
 661 tccggccat cggccggcc tggccggccat gcaatgtgt ggcggccgt gcaatgtgg
 721 gacccatc cggccatc tggatcaaca tggggccaa cggccgggg aagacggccgt
 781 cggccggccatc cggccggccaa gagcaaggcg gggccgtt ccgggggggg tccgtgtcg
 841 cccatgtgt cggccggccatc aagccatc cccggccaa gatccggccg gggatggatgaa
 901 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 961 ggatccatc tggccatc tggatccatc cggatccatc aaacccatc tttttttttt
 1021 tggatccatc tggccatc tttttttttt tttttttttt tttttttttt tttttttttt
 1081 gcaatccatc tcaatccatc tttttttttt tttttttttt tttttttttt tttttttttt
 1141 tcaatccatc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 1201 gcaatccatc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 1261 tcaatccatc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt

//

LOCUS XXXXX 1450 bp mRNA PRI 20-OCT-1997
 DEFINITION Homo sapiens small cell lung cancer vasopressin receptor subtype 1b
 mRNA, complete cds.

ACCESSION AF030512

KEYWORDS

SOURCE human.

ORGANISM Homo sapiens
 Eukaryotae; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;
 Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 1450)

AUTHORS Sugimoto,T., Saito,M., Mochizuki,S., Watanabe,Y., Hashimoto,S. and
 Kawashima,H.

TITLE Molecular cloning and functional expression of a cDNA encoding the
 human V1b vasopressin receptor

JOURNAL J. Biol. Chem. 269, 27088-27092 (1994)

REFERENCE 2 (bases 1 to 1450)

AUTHORS Du,J., Fay,M.J., Longo,K.A. and North,W.G.

TITLE Human vasopressin receptor subtype 1b in small cell carcinoma of
 the lung

JOURNAL Unpublished

REFERENCE 3 (bases 1 to 1450)

AUTHORS Du,J., Fay,M.J., Longo,K.A. and North,W.G.

TITLE Direct Submission

JOURNAL Submitted (20-OCT-1997) Physiology, Dartmouth Medical School, 1

Medical Center Drive, Lebanon, NH 03756, USA

COMMENT de Keyzer, Y. FEBS Lett. 356, 215-220, 1994.

FEATURES Location/Qualifiers

source 1..1450

/organism="Homo sapiens"

/note="cell type, human small-cell carcinoma of the lung,
 cell line, NCI-H82;"

gene 1..1450

/gene="small cell vasopressin subtype 1b receptor"

CDS 124..1398

/gene="small cell vasopressin subtype 1b receptor"

/codon_start=1

/product="small cell vasopressin subtype 1b receptor"

/translation="MDSGPLWDANPTPRGTL SAPNATT PWLGRDEELAKVEIGVLATV

LVLATGGNLAVLLTGQLGRKRSRMHLFVLHLALTDLAVALFQVLPQLLWDITYRF
 QG

PDLLCRAVKYLQVLSMFASTYMLLAMTLDRYLAVCHPLRSLQQPGQSTYLLIAAPW
 LL

AAJFSLPQVFIFSLREVIQGSGVLD CWADFGFPWGPRAYLTWTTLAIFVLPVTMLTA
 C

YSLICHEICKNLKVKTQAWRVGGGGWRTWDRSPSTLAATTRGLPSRVSSINTISR
 AK

11

LOCUS XXXXX 1201 bp mRNA PRI 21-OCT-1997
DEFINITION Homo sapiens vasopressin V2 receptor mRNA, complete cds.

ACCESSION AF030626

KEYWORDS

SOURCE human.

ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;
Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 1201)

AUTHORS Birnbaumer, M., Seibold, A., Gilbert, S., Ishido, M., Barberis, C.,
Aniaramian, A., Brabet, P. and Rosenthal, W.

TITLE Molecular cloning of the receptor for human antidiuretic hormone

JOURNAL Nature 357, 333-335 (1992)

REFERENCE 2 (bases 1 to 1201)

AUTHORS Fay, M.J., Du, J., Yu, X. and North, W.G.

TITLE Evidence for Expression of Vasopressin V2 receptor mRNA in Human

Lung

JOURNAL Peptides 17, 477-481 (1996)

REFERENCE 3 (bases 1 to 1201)

AUTHORS Du, J., Fay, M.J., Yu, X. and North, W.G.

TITLE Direct Submission

JOURNAL Submitted (21-OCT-1997) Physiology, Dartmouth Medical School, 1

Medical Center Drive, Lebanon, NH 03756, USA

COMMENT Seibold, A. Am. J. Hum. Genet. 51, 1078-1083, 1992.

FEATURES Location/Qualifiers

source 1..1201

/organism="Homo sapiens"

/note="cell type, human fetal lung;"

gene 1..1201

/gene="Human lung vasopressin V2 receptor"

CDS 33..1148

/gene="Human lung vasopressin V2 receptor"

/codon_start=1

/product="Human lung vasopressin V2 receptor"

/translation="MLMASTTSAVPGHPSLPSLPSNSSQERPLDTRDPLLARAELALL

SIVFVAVALSNGLVLAALARRGRRGHWAPIHVFIGHLCLADLAVALFQVLPQLAW
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DRFRGPDALCRAVKYLQMVGMYASSYMLAMTLDRHRAICRPMILAYRHGSGAH
WNRPV

LVAWAFSLLISLPQLFIFAQRNVEGGSGVTDCWACFAEPWGRRTYVTWIALMVFV
APF

LGIAACQVLIFREIHASLVPGPSEPGGRRGRRTGSPGEGAHVSAAVAKTVRMTL
VI

VVVYVLCWAPFFLVQLWAAWDPEAPLEGAPFVLLMLLASLNSCTNPWVYASFSSS
VSS

ELRSLLCCARGRTPPSLGPQDESCTTASSSLAKDTSS"
BASE COUNT 174 a 407 c 375 g 245 t
ORIGIN

1 caggccccca gaacaccgc cccagccca ccacgcacat ggccgcacc accccgcig
61 tgcclgggca tccctcicig cccagccgc ccagcaacag cagccaggag aggccacig
121 acacccggga cccgcgcia gcccggcgg agcggcgt gctccatia gcttgggg
181 cttggccctt gagcaatggc cttggcgtgg cggcccttgc tggcggggcc cggcggggcc
241 actgggcacc caaacgc tcaatggcc acitggcctt ggccgaccig ggcggggc
301 tgcacatgt gctggccca g cttggccggaa ggccacccga ccgcacccgt gggccagaatg
361 cccctggcggccat ggcggccat gatctgcaga tggggccat gtaatccccc tccatca
421 tccctggccat gacgcgtggac cggccaccggc ccacgcgcg tccctggcgt ggcggcc
481 atggaaatggg ggcgtacgg aaccggccgg tgcgtggc tggggccac tgcgtcc
541 tcaagccgc cccgcaccc acacccgcggc agcgcacat gggggggc gggggggca
601 tgcacgcgt ggcgtggcc tggggccgtt gggccgtt caccatgc accggatgt
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721 gggagatca tggccatgc tggccaggcc caatcggatgg gccgggggg cggccggagg
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901 tgcacgttgc ggcggccgg gacccgggg caccatggat gggggccccc ttttttttt
961 tcaatgttgc ggcggccgc tcaatgttgc cccatccctgg gttttttttt ttttttttt
1021 gcaatgttgc tcaatgttgc tcaatgttgc cccatccctgg gttttttttt ttttttttt
1081 ggcggccccc cc22atgttgc tccatccatca cccatccctggcc aatggatgtt
1141 caatgttgc tcaatgttgc tcaatgttgc tcaatgttgc tcaatgttgc tcaatgttgc
1201 c

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LOCUS XXXXX 1298 bp mRNA PRI 08-OCT-1998
DEFINITION Homo sapiens breast cancer vasopressin receptor subtype 1a mRNA.

complete cds.

ACCESSION XXXXX

KEYWORDS

SOURCE human.

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;
Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 1298)

AUTHORS North,W.G., Fay,M.J., Longo,K.A. and Du,J.

TITLE Expression of all known vasopressin receptor subtypes by small cell
tumors implies a multifaceted role for this neuropeptide

JOURNAL Cancer Research 58, 1866-1871 (1998)

REFERENCE 2 (bases 1 to 1298)

AUTHORS North,W.G. and Du,J.

TITLE Breast cancer cells express normal forms of all vasopressin
receptor subtypes plus an abnormal v2 receptor

JOURNAL Unpublished

REFERENCE 3 (bases 1 to 1298)

AUTHORS Du,J. and North,W.G.

TITLE Direct Submission

JOURNAL Submitted (08-OCT-1998) Physiology, Dartmouth Medical School, 1
Medical Center Drive, Lebanon, NH 03756, USA

FEATURES Location/Qualifiers

source 1..1298

/organism="Homo sapiens"

/db_xref="taxon:9606"

/note="Breast cancer cell line MCF-7;"

gene 1..1298

/gene="Breast cancer vasopressin receptor subtype 1a"

mRNA 24..1280

/gene="Breast cancer vasopressin receptor subtype 1a"

CDS 24..1280

/gene="Breast cancer vasopressin receptor subtype 1a"

/codon_start=1

/product="Breast cancer vasopressin receptor subtype 1a"

/translation="MRLSAGPDAGPSGNSSPWWPLATGAGNTSREAEALGEGNGPPRD

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QQPARRSRLMIAAAWVLSFVLSTPQYFVFSMIEVNNVTKARDCWATFIQPWGSRAYVT

WMTGGIFVAPVVLGTCYGFICYNIWCVNRGKTASRQSKGAEQAGVAFQKGFLAPCV

SSVKSISRAKIRTVKMTFVITAYIVCWAPFFIQQMWSVWDPMSVWTESENPTITITA

LLGSLNSCCNPWIYMFSGHLLQDCVQSFPCQNMKEKFNKEDTDSMSRQTFYSNNR

SPTNSTGMWKDSPKSSKSIFPVT"

BASE COUNT 258 a 399 c 364 g 277 t

ORIGIN

1 cgagtaggag ctgcgtggac agcatgcgt 1ccgcgcgg tccgcacgcg gggccctcg
61 gcaacccagg cccatgggg cctctggca cccggcgctgg caacacaagc cgggaggccg
121 aagccctcggtt ggaggggcaac gcccacccga gggacgtggc caacgaggaa ctggccaaac
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301 tcaacccgtt ccgcgtggcc gggccatcgc tccggatgtt gcccggaaatcgttggaca
361 tcaaccttccgcgtt ccgcgtggcc tgcgtggccgtt ggttggaaatcgcgg
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541 ccgcgtgggtt gtcgtggccatcgc tgcgtggccatcgc tccatgtatcgc
601 aggttacaaatcgc tcaacccgc gcccggccatcgc tccatccatcg cccggggatc
661 ctgcgtggccatcgc tcaacccgc gcccggccatcgc tccatcg tccatcg
721 tcaatcgccatcgc tcaacccgc tcaatcgatcgc tcaatcgatcgc
781 ccgcgtggccatcgc tcaacccgc cccgcgcggcc tccgcgcgc tcaatcgatcgc
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1021 tcaatcgatcgc tcaacccgc tcaatcgatcgc tcaatcgatcgc tcaatcgatcgc
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//

